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Immuno-pcr Detection Of Lyme Borreliosis

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IMMUNO-PCR DETECTION OF LYME BORRELIOSIS

by

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ABSTRACT

Lyme borreliosis, more commonly referred to as Lyme disease, is the fastest growing zoonotic disease in North America with approximately 30,000 confirmed cases and 300,000 estimated infections per year. In nature, the causative agent of Lyme disease, the bacterium *Borrelia burgdorferi*, cycles between *Ixodes sp.* ticks and small mammals. Humans become infected with Lyme disease after being bitten by an infected tick. The primary indicator of a *Borrelia burgdorferi* infection is a bull's eye rash typically followed by flu-like symptoms with treatment consisting of a 2-4 week course of antibiotics. If not treated, later stages of the disease can result in arthritis, cardiovascular and neurological symptoms. Diagnosis of Lyme disease is challenging and currently requires a complex laboratory diagnostic using indirect detection of host-generated antibodies by a two-tiered approach consisting of an enzyme linked immunosorbent assay (ELISA) followed by IgM and IgG immunoblots. Although two-tier testing has provided an adequate approach for Lyme disease diagnosis, it has weaknesses including subjective analysis, complex protocols and lack of reagent standardization. Immuno-PCR (iPCR) is a method that combines ELISA-based detection specificity with the sensitivity of PCR signal amplification and has demonstrated increased sensitivity for many applications such as detection of disease biomarkers but has yet to be applied for diagnosis of Lyme disease.

Herein, using iPCR and recombinant *B. burgdorferi* antigens, an assay for both the direct and the indirect detection of Lyme disease was developed

and demonstrated improved sensitivity for detection of *B. burgdorferi* antibodies using a murine model. Moreover, we present evidence using human Lyme disease patient serum samples that iPCR using both multiple antigens and a unique single hybrid antigen is capable of achieving increased sensitivity and specificity compared to existing methodology. These data represent the first demonstration of iPCR for Lyme disease diagnosis and support the replacement of two-tier testing with a more simplified and objective approach.

To my family (two and four legged) for their never ending support in all I do and to my wife, love of my life, partner in crime and best friend Adriana who makes everything I do pale in comparison to her overwhelming love, patience and vision. With you beside me, anything is possible.

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LIST OF ABBREVIATIONS

BSK	Barbour-Stoenner-Kelly
CDC	Centers for Disease Control and Prevention
Cfu	colony forming units
CI	confidence interval
Cq	quantification cycle
CRASPS	complement regulator-acquiring surface proteins
CV	coefficient of variation
ELISA	enzyme linked immunosorbent assay
EM	erythema migrans
GST	glutathione-S-transferase
iPCR	immuno-PCR
kbp	kilobase pairs
kDA	kilodaltons
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PTLDS	Post-treatment Lyme Disease Syndrome
SD	standard deviation
TBST	Tris buffered saline/0.05% tween, pH 7.6
UCF	University of Central Florida

CHAPTER ONE:
INTRODUCTION

Lyme Disease Background

History

Beginning in the early 1970s, a cluster of apparent rheumatoid arthritis cases occurring primarily among children and some adults in the areas surrounding Lyme, Connecticut captured the attention of the public health community. A surveillance of the town resulted in 39 children diagnosed with reoccurring symptoms of large joint swelling and pain with some episodes lasting for extended periods of time [1]. An additional twelve adults either related or living in close proximity to the arthritic children were also diagnosed with signs and symptoms similar to those seen in the juvenile cases. The investigation excluded juvenile rheumatoid arthritis as the cause of the epidemic on the basis that the prevalence of symptoms (particularly arthritis) was 100 times greater than that expected for juvenile rheumatoid arthritis for the surrounding Connecticut communities. The disease was initially named Lyme arthritis to indicate the town and initial symptom observed. Following an expansion of the clinical symptoms to involve both neurological and cardiac symptoms, the name was finally changed to Lyme disease [2].

The growing investigation revealed that about 25% of the patients had developed an erythematous cutaneous lesion that appeared to expand into a reddish rash composed of concentric rings similar in appearance to a ‘bull’s eye’. The lesion typically appeared weeks before the onset of disease

symptoms and resembled a similar lesion that had been described much earlier in Europe that was associated with being bitten by an *Ixodes rincinus* tick [3]. Investigators followed patients with cutaneous lesions [4] and subsequently observed a range of symptoms including arthritis, neurological and cardiac abnormalities [5]. The addition of neurological and cardiac involvement underscored the complex manifestation of the disease that appeared to involve multiple organ systems [2, 5].

The evidence presented for the European rash and its association with a tick bite led to a similar hypothesis in the Connecticut cases that a local *Ixodes* species was associated with the disease [6]. This conclusion was also further supported by the fact that many of the children diagnosed with arthritis either lived or routinely played adjacent to wooded areas where *Ixodes* ticks were commonly found. It was also noted that the children's initial symptoms typically started during the summer months, which coincided with the peak of tick season. Many of the children reported having a skin rash similar in description to an erythema migrans (EM), or 'bull's-eye' rash prior to developing arthritis that was often associated with a tick bite [1]. Another important piece of supporting evidence was that patients exhibiting the EM rash that were treated with penicillin showed a shortened duration of the rash and lessened subsequent arthritis [7]. The affective treatment of the disease with antibiotics provided a key piece of evidence that a bacterial infection was most likely associated with disease progression [7].

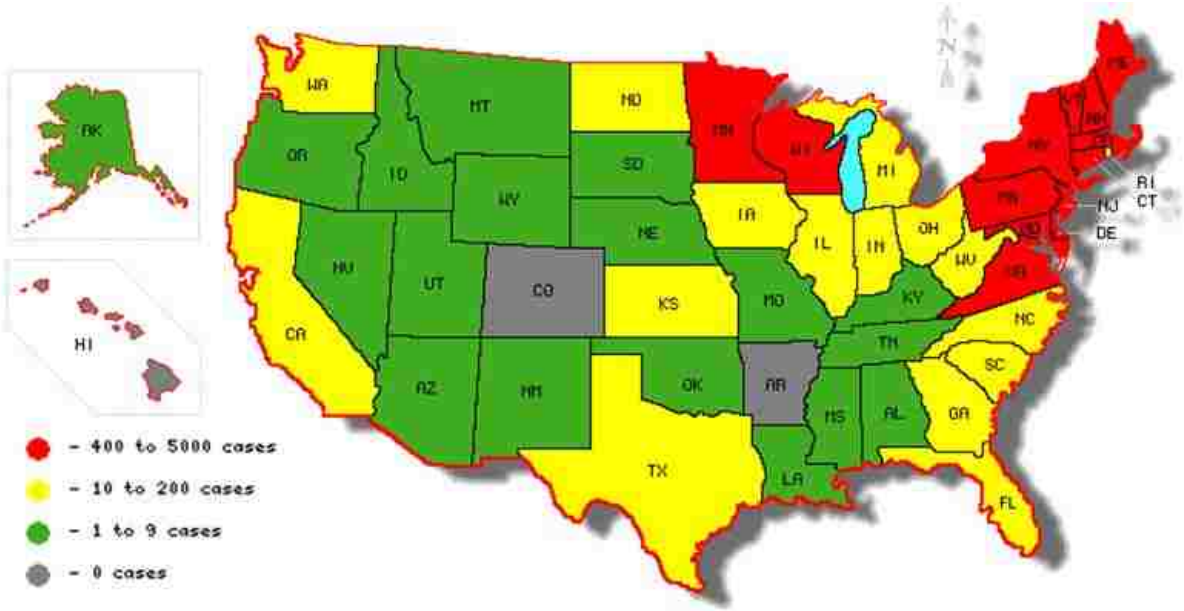
Similar studies at the time surrounding a suspected epidemic of babesiosis caused by a parasite in Shelter Island, New York provided the next

critical step in unravelling the cause of the disease. Dark field microscopy of organisms isolated from *Ixodes scapularis* adult ticks collected as part of a serosurvey for the Shelter Island cases identified coil shaped bacteria known as spirochetes in the samples. These bacteria were further cultured and confirmed in a spirochete specific medium thus confirming the presence of the organism. Serum from individuals exhibiting symptoms of Lyme disease from the Connecticut group were then tested using the tick isolated organisms and showed strong sero-reactivity to the newly cultured spirochetes [8]. This indicated the Lyme disease patients had most likely been infected with the same organism. The final piece of evidence was uncovered when the same tick isolated spirochetes that exhibited sero-conversion in Lyme patients were also isolated from skin samples of patients with the EM rash [9]. Further investigations also isolated the same organism from white-footed mice in the local Connecticut communities [10] thus identifying the main reservoir of the bacterium [11]. With the reservoir identified and reisolation of the causative bacteria from human patients exhibiting the characteristic EM, a strong explanation for the arthritis epidemic observed in areas surrounding Lyme, Connecticut had been determined. The future of Lyme disease research would now focus on the biology of the bacteria, transmission, human infection and diagnosis of the disease.

Lyme Disease Infection

Historically, there have been approximately 30,000 confirmed cases of Lyme disease per year seen across almost every state with the majority of

cases concentrated in the Northeastern and Midwestern states [12]. Representative statistics for the CDC confirmed cases of Lyme disease in 2011 are depicted by state in Figure 1. More recently, the CDC has revised these statistics to suggest an approximate estimate of 300,000 infected cases per year [13].



Pennsylvania	4739	Delaware	767	Iowa	72	Alaska	9	Mississippi	3
New Jersey	3398	Virginia	756	Ohio	36	Montana	9	Nevada	3
New York	3118	Vermont	476	Georgia	32	Oregon	9	New Mexico	2
Wisconsin	2408	Illinois	194	Texas	28	Arizona	8	Oklahoma	2
Connecticut	2004	Rhode Island	111	South Carolina	24	Nebraska	7	South Dakota	2
Massachusetts	1801	West Virginia	107	North Dakota	22	Utah	6	Louisiana	1
Minnesota	1185	Michigan	89	North Carolina	18	Missouri	5	Wyoming	1
Maryland	938	Indiana	81	Washington	17	Tennessee	5	Arkansas	0
New Hampshire	887	California	79	Kansas	11	Idaho	3	Colorado	0
Maine	801	Florida	78	Alabama	9	Kentucky	3	Hawaii	0

Figure 1. United States 2011 CDC confirmed Lyme disease cases. Cases are determined based on the state of residence and not the state where the infection was contracted. Case number ranges are depicted based on the color scale with the exact number of cases listed by State in the bottom table [14, 15].

The primary indicator of Lyme disease infection by the *B. burgdorferi* spirochete is the appearance of an EM or 'bull's eye' rash that is typically observed in approximately 75% of cases [16]. An oval or circular rash can be observed over days or weeks following a tick bite that begins as a small red spot at the site of the bite [6]. More specific to Lyme disease, the rash continues to form a red ring surrounding a clear area with a red spot in the center very similar in appearance to the concentric circles of an archery target. The rash, which typically occurs at the site of the tick bite, can take a few weeks to fully present in infected patients and can vary in size from a silver dollar to covering the entire surface of the torso [17]. Following the spread of infection, additional similar rashes both with and without a bull's eye center can manifest at additional locations distal to the original tick bite and indicate dissemination of the Lyme disease spirochete [18]. Although the bull's eye rash is the hallmark symptom of Lyme disease, other symptoms similar to common viral infections such as fever, aches, stiffness and fatigue can accompany infection and can last for longer periods of time than other common infections [19]. Assuming the infection goes untreated and following organism dissemination, additional symptoms can be observed including arthritis, nervous system complications and more rarely can involve cardiac symptoms as well [20]. In even rarer cases, *B. burgdorferi* infection has been documented to cause severe fatigue [21], eye inflammation [22] and hepatic liver disease [23].

Recommended treatment for Lyme disease is a 2-4 week course of antibiotics [24]. Although typically successful at resolving the infection,

approximately 15% of patients will continue to experience symptoms of muscle and joint aches or fatigue following treatment [25]. Pain symptoms can sometimes persist for 6 months or more following treatment after which the disease is often referred to as 'Chronic Lyme Disease' due to the inability to resolve symptoms with standard antibiotic treatment [25]. If symptoms persist following treatment the condition is more appropriately being referred to as 'Post-treatment Lyme Disease Syndrome' (PTLDS) [26]. Although the cause of PTLDS remains elusive, it has been suggested that unresolved symptoms result from residual tissue damage due to overstimulation and/or self-recognition of the immune system resulting from the original spirochete infection [27]. Lyme disease is suspected to be similar to other human infections including *Chlamydia* [28] and *Campylobacter* [29] in which similar 'auto-immune' reaction based diseases have been observed following successful treatment of the infection. In direct contrast to the residual tissue damage theory, additional theories have been postulated that unresolved symptoms are a reflection of persistent infection with *Borrelia burgdorferi* that was not cleared with an initial course of antibiotics [30]. Select groups of health care providers have advised their patients that based on this theory their conditions warrant a longer term regimen of antibiotic treatment beyond the recommended 2-4 week course of treatment [31]. This is in direct contradiction to studies that have demonstrated that Lyme disease patients who received prolonged courses of antibiotics did not result in an outcome significantly better than patients treated with a placebo [32, 33]. Regardless of the cause of PTLDS, it continues to be a controversial issue [34] that

underscores the importance and strong need for improved Lyme disease diagnosis.

Ixodes scapularis Life Cycle

The only known insect vectors of Lyme disease in the United States are *Ixodes scapularis*, commonly known as the deer or black-legged tick in the Eastern part of the United States and the closely related *Ixodes pacificus* or western black-legged tick in the Western part of the United States [35]. Approximate distributions for each species are shown in Figure 2. *I. scapularis* and *I. pacificus* are both hard-bodied ticks that display a two-year life cycle that is dependent on a fresh blood meal from a vertebrate to advance to the next stage in each cycle [36].

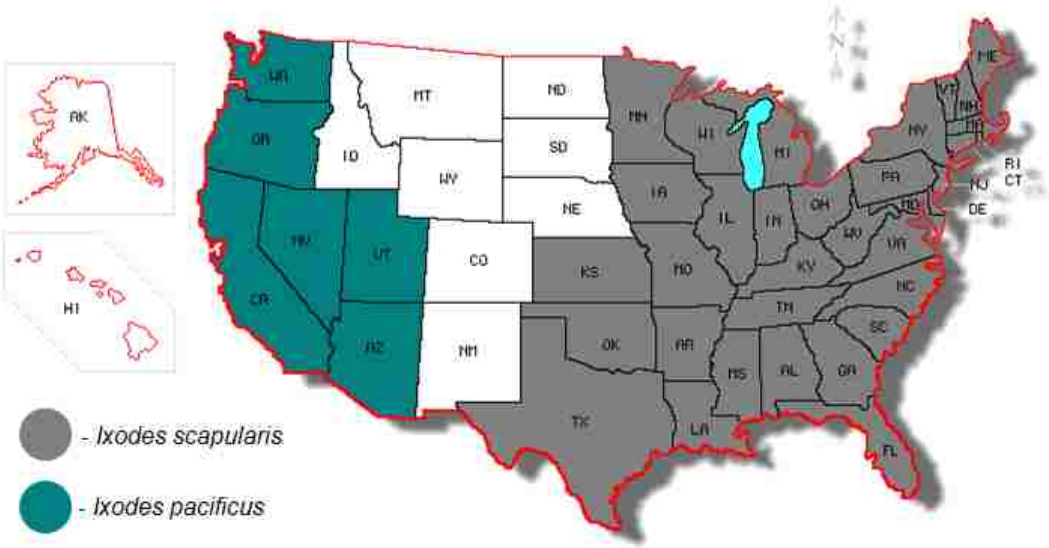


Figure 2. Black-legged tick distribution. States where each species can be found are shown [15, 37].

The deer tick proceeds through three distinct stages of growth over its life cycle that includes the larval, nymph and adult stages (Figure 3). It takes approximately two years for the tick to proceed from an egg through all three developmental stages, reproduce, subsequently lay eggs and ultimately expire at the end of the cycle [38]. The tick life cycle begins with egg hatching and emergence of larvae in spring. This is then followed by development into nymphs the ensuing year after the first blood meal and finally by development into adults the subsequent year after the second blood meal [39]. *B. burgdorferi* infection is acquired from infected nymphs or adults with uninfected larvae posing no danger to humans due to the absence of the infecting organism [40]. People are usually able to notice attached and feeding adult ticks making them easy to remove prior to spirochete infection. A large number of Lyme disease cases originate from feeding nymphs that are smaller and more difficult to notice. Hence, the majority of Lyme disease cases are reported during the summer months with very few cases reported in the spring and fall [39].

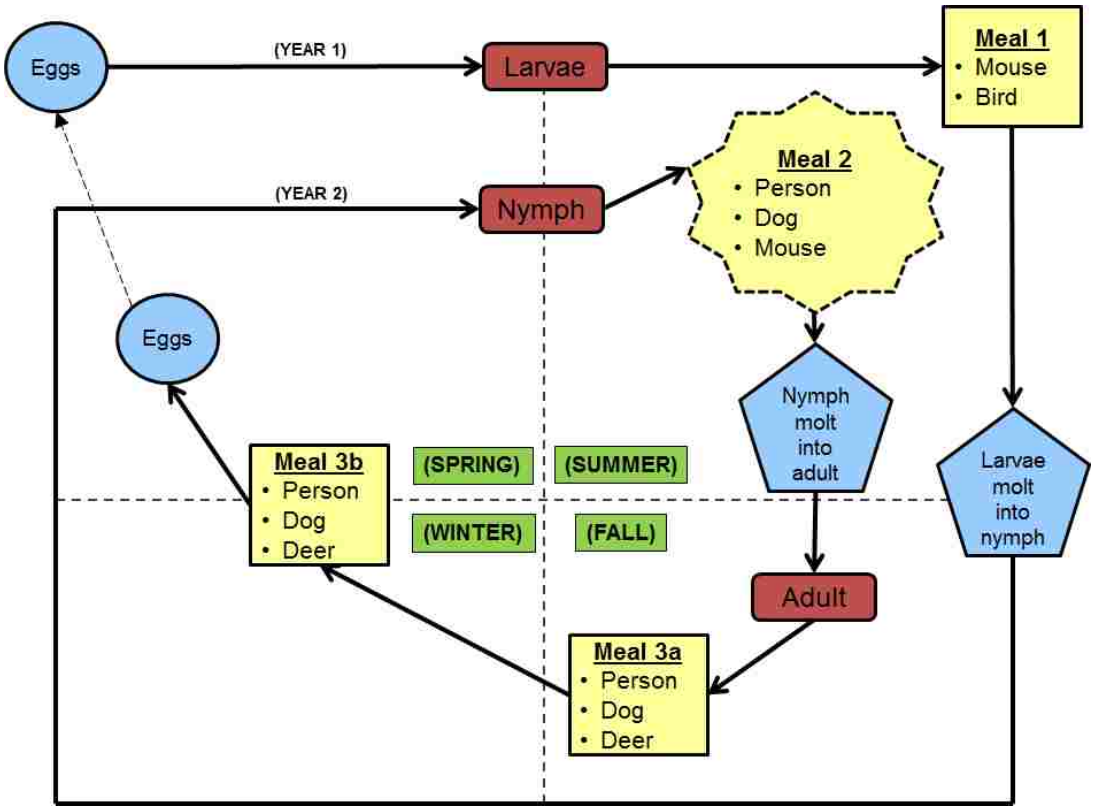


Figure 3. Black legged tick life cycle. The two-year life cycle of the *Ixodes* tick begins with eggs laid by the female in spring that proceed to hatch into larvae that partake of their first blood meal in the summer. This is followed by molting into the nymph form that then overwinters and remerges the following spring and again partakes of a blood meal followed by molting into the adult form that feeds a third time in late fall or early spring of the second year and followed by egg laying in late spring and a repetition of the cycle.

The larval form comprises the first stage of the tick infectious cycle [41]. The adult female *Ixodes* tick lays its eggs in the spring, which then hatch into larvae during late summer. The larvae, which are no bigger than a pin head, begin to peak in activity in late August [42]. A small mammal or bird that contacts the awaiting larvae on the ground then becomes the first host for the tick in the first stage of its life cycle. The larva uses its mouth-parts to attach to the host and then begins its first blood meal. Over a period of 3-5 days the tick swells with the blood meal from the host [38]. It is at this stage

that the spirochete can make its first transfer between hosts. If the mammal or bird had been previously infected with the organism from a subsequent tick bite, the larva would now become infected with the Lyme disease spirochete. The spirochete utilizes large numbers of wild infected hosts (such as white-footed mice) as a reservoir that can continue to infect new larvae through each transmission cycle [43]. Although mice and other small mammals are the principal reservoir for *B. burgdorferi*, ground-feeding birds can serve as natural reservoirs as well [44]. Since larval ticks require a blood meal to become infected, freshly hatched larvae cannot transmit Lyme disease to animals or humans and hence pose no danger for passage of the disease. Instead, larvae act as the first stage of the infectious life cycle through feeding on 'reservoir' hosts. Once a larvae has fed, it will not feed again until the next stage in its life cycle [45].

The second stage of the tick life cycle poses the largest threat of human infection and is characterized by the nymphal form of the tick [45]. After completing their blood meal at the end of the first stage in their life cycle, the larvae disassociate from their host and return to the ground where they molt and metamorphose into nymphs. This occurs at the end of the fall after which the nymphs become dormant through the winter and early months of spring. Typically around May, the nymphs awaken from their inactive overwinter state, climb onto nearby vegetation and await the opportunity to interact with a new small mammal or bird host as they pass by [46]. The nymph takes the opportunity to attach to the host animal on which it will feed for approximately five days. During feeding, the nymph will engorge with

blood and swell many times its original size. If the nymph had been infected during its blood meal in the larval stage, it can transmit infectious *B. burgdorferi* to its new host after about 48 hours of feeding [47]. Similar to the larval stage, if the nymph is uninfected at this point in its life cycle, it can become infected if its host already carries the Lyme disease pathogen from a previous infectious tick bite. Surveys of ticks in the highly endemic Northeast and upper Midwest have found that about 25% of nymphs contain and can transmit *B. burgdorferi* [48]. Regrettably, because of frequent outdoor activity during the spring months, humans will often come into contact with populations of infected nymphs during their peak activity, which can occur from late May to the end of July. Nymphs normally feed on small mammal and bird hosts but will feed upon humans, pets and other domestic animals if the opportunity presents itself. Although not as small as larvae, nymphs reach the size of about a poppy seed [39] which makes them difficult to notice when they are attached to visible areas on the skin and even more difficult to locate in less conspicuous areas of the body such as the scalp or armpits. Hence, nymphs are responsible for transmission of a large proportion of human Lyme disease cases [45].

The third and last stage of the tick life cycle involves progression to the adult form followed by reproduction. After finishing its blood meal, the nymph releases from its host, falls to the ground and begins the process of molting or transforming into the adult and reproductive stage of its life cycle [49]. After completing development in early fall, the adult tick climbs and perches on vegetation a few feet off the ground and awaits another host such as a deer or

other large mammal to complete its last blood meal. Late October and early November mark the peak of activity for adult tick feeding [50]. Due to the increased potential for infection at either the larval or nymph stage, a higher percentage of adult ticks surveyed in endemic areas have been found to be infected with *B. burgdorferi* with as much as 50% of the population in the Northeast testing positive for the Lyme disease spirochete [51]. In contrast to infection by bites from nymphs, fewer cases of Lyme disease are associated with adult tick bites because of their larger size. When fully engorged with a blood meal, adults can reach the size of a small grape and hence are much more likely to be detected and removed prior to the 36 hours required for disease transmission [52].

At the end of the fall and beginning of the winter months, both fed and unfed adult ticks migrate underneath ground cover and surface vegetation to overwinter. Adult ticks become inactive in temperatures below 45° F which means winter temperatures in the endemic Northeast and upper Midwest states keep ticks inactive in a dormant state until temperatures rise again in late February to the beginning of March [53]. As the temperature warms, adult ticks will again resume their attempts to acquire a host blood meal prior to actively seeking a mate for reproduction which peaks in typically March to the beginning of April [41]. Tick mating in the spring can occur either while attached to a host or following a blood meal and is followed by the laying of eggs by the female underneath leaf litter and other ground cover. An adult female tick can lay up to 3,000 eggs in a litter after which she will die and the eggs will hatch later that summer completing the two-year life cycle [54].

Borrelia burgdorferi Biology

Borrelia burgdorferi, the causative agent of Lyme disease in the United States is a spirochete bacterium that appears gram negative by safranin staining, but is not typically classified as either gram positive or negative [55]. The other two Lyme disease causing species of the genus *Borrelia* are *B. garinii* and *B. afzelii*, which are responsible for the majority of cases in Europe [56]. Of the three species, *B. burgdorferi* is unique in that it has an extensive distribution encompassing most of the northern hemisphere including both North American and Europe [57]. *B. burgdorferi* is transmitted by ticks, requires small mammals as hosts to complete its life cycle and causes disease in humans and other mammals by causing an inflammatory response. The bacteria are able to adapt to the mammalian environment by dramatically up-regulating or down-regulating gene expression as it is transmitted from the infected tick to the mammalian host [45].

B. burgdorferi causes a primary infection by moving into the bloodstream of the infected mammal and colonizing different tissue sites. Through chemotaxis and unique mechanisms of motility, the organism continues to disseminate throughout the body leading to a more advanced stage of infection [58]. A number of *B. burgdorferi* proteins have been implicated in host cell adherence through interactions with surface proteins and extracellular matrix components. As a result of these proteins, the organism is capable of blood vessel penetration, adherence to endothelial cells and interacting with a number of different tissues [59]. For example, *B. burgdorferi* stimulates plasmin on the bacterial surface and has the capability

to induce host protease production which aides in its dissemination and ultimately leading to tissue damage and inflammation [60]. *B. burgdorferi* infection can lead to arthritic, dermatological, cardiovascular and neurological symptoms across the multiple stages of infection in spite of low numbers of microorganisms in tissues [61]. Unique mechanisms to evade the immune system allow the spirochete to persist for long periods of time post infection in the presence of strong antibody and cellular host responses [62]. Interestingly, the pathogen has not been shown to produce any toxins to aide its persistence and disease manifestation over either short or extended time periods [45].

Much of the adaptive nature of the organism is attributed to its unique genome [63]. Sequencing of the *B. burgdorferi* genome confirmed the presence of an approximately 900 kilobase pairs (kbp) linear chromosome and an extra-chromosomal complement of linear and circular plasmids in the range of 55-220 kbp [64]. The genome sequence for multiple strains of all three Lyme disease causing *Borrelia* species (*B. burgdorferi*, *B. afzelii*, *B. garinii*) [65, 66] as well as multiple related species (*B. hermsii*, the causative agent of relapsing fever) [67] have been completed or are currently underway. A large number of housekeeping genes, which are conserved in sequence and organization across the genus, are carried on the chromosome. The up to 22 circular and linear plasmids, which can vary in number by species/isolate and are much more variable in sequence than the chromosome, encode a large number of differentially expressed surface proteins known to interact with the *B. burgdorferi* tick vector and mammalian

host [68]. One of the more studied strains is the *B. burgdorferi* B31 type strain, which possesses nine circular and twelve linear plasmids and a small linear chromosome. Beyond coding gene sequences, *B. burgdorferi* plasmids are unusual in that they contain a large number of paralogous and pseudogene sequences as well as genes essential for host infection [63]. In addition, many genes have been experimentally deleted from *B. burgdorferi* plasmids with no resulting detectable phenotype [69].

B. burgdorferi has been shown to modulate its expression profile in response to the numerous environmental cues encountered throughout its infectious cycle and can therefore demonstrate variation in antigen presentation throughout infection [70-86]. For example, during early stages of infection *B. burgdorferi* is capable of attachment to host tissues through expression of different adhesins that can bind integrins [87], fibronectin [88, 89], proteoglycans [90], glycosaminoglycans [91] and laminin [92]. *B. burgdorferi* has also been shown to express proteins known as complement regulator-acquiring surface proteins (CRASPs) that bind factor H and prevent the spirochete from being killed by the complement arm of the innate immune system [93]. Crucial to clearance of the pathogen in the mammalian host is development of a humoral or antibody response against specific *B. burgdorferi* antigens [94]. Several well-known immunodominant antigens are expressed by *B. burgdorferi* early in disease including flagellin [95], OspC [96] and BmpA [97]. As the disease progresses further resulting in spirochete dissemination, additional antigens are known to illicit an immune response as determined by immunoblot [98, 99]. *B. burgdorferi* has been shown to also

utilize the VlsE lipoprotein which undergoes antigenic switching through a recombination mechanism to further avoid the immune system [100] and consequently this protein has proved useful as an immunodiagnostic target [101]. More recent studies have focused on screening large numbers of *B. burgdorferi* proteins using protein arrays with the aim of discovering antigens that illicit a strong immune response [102] toward the ultimate goal of contributing to development of the next generation of Lyme disease diagnostics.

Current State of Lyme Diagnostics

Due to the fact that Lyme disease can present with symptoms like fever, pain and fatigue which are very similar to other common illnesses, treating physicians may have difficulty diagnosing a *B. burgdorferi* infection based on these symptoms alone [103]. Although the 'bull's eye' rash or EM is the only unique Lyme disease symptom it is only typically detected in about 75% of patients infected with *B. burgdorferi* and can be difficult to detect based on size and/or location on the head or torso [17]. To further complicate the diagnosis, people may not recall or detect the actual tick bite required for transmission of the pathogen. This is typically due to the fact that many people are bit by the *Ixodes* tick in the nymph stage, which is small and difficult to detect [104]. Assuming symptoms match that of Lyme disease and in the absence of an EM rash, physicians will then rely on a detailed medical history to rule out additional potential infectious agents and/or diseases. The physician will look for specific details relating to *B. burgdorferi* exposure

including if the person frequents areas endemic for Lyme disease, if a tick bite was detected or suspected and what part of the year symptoms first appeared (i.e., summer months). This information is then combined with a thorough physical examination for evidence of tick bites and laboratory diagnostic tests that are used to further aide in diagnosis [105].

Two-Tier Laboratory Diagnostic

The accepted method of Lyme disease laboratory diagnosis is indirect detection of host generated antibodies to *Borrelia burgdorferi*, the causative agent of the disease [106]. It takes days to weeks for an individual infected with *B. burgdorferi* to generate sufficient IgM or IgG antibody titers, respectively, against the bacteria to be detected using laboratory based diagnostics [107]. The current accepted method of serologic testing for Lyme disease utilizes a 2-tier approach that was established in 1995 [106]. The method entails testing of a serum sample using a first-tier enzyme linked immunosorbent (ELISA) assay employing *B. Burgdorferi* whole cell sonicate or recombinant antigens that results in high sensitivity but somewhat reduced specificity. A negative first-tier result means no further testing of the specimen is recommended. A positive first-tier result is followed by retesting of the serum sample by distinct IgM and IgG immunoblots (or western blots) that typically employ *B. burgdorferi* whole cell sonicate. An individual is considered to be diagnostically positive for Lyme disease only if the ELISA (first-tier) and the immunoblot (second-tier) are both positive.

Each step of two-tier testing requires different analytical criteria and subsequent results are either quantitative (ELISA) or qualitative (immunoblot) based on the tier. The first-tier ELISA measures a quantitative immune response to typically a single *B. burgdorferi* antigen or immunogenic peptide or a limited number of recombinant *B. burgdorferi* antigens. Increased values are typically correlated with the numbers of *B. burgdorferi* antibodies in the serum and provide a measure of immune response level. First-tier results are categorized as positive, equivocal or negative based on pre-established value ranges [108]. Second-tier testing using IgM and IgG immunoblots provides a qualitative measurement of antibody response and typically employs the use of *B. burgdorferi* whole cell sonicate [99]. There are a small number of kits approved for use as a second-tier Lyme disease diagnostic that employ multiple recombinant *B. burgdorferi* antigens [109]. Second-tier IgM immunoblot results are considered positive if two of three *B. burgdorferi* specific bands (23, 39 and/or 41 kDa) are detected above a particular signal threshold [106]. Second-tier IgG immunoblot results are considered positive if five of ten *B. burgdorferi* specific bands (18, 21, 28, 30, 39, 41, 45, 58, 66 and/or 93 kDa) are detected [106]. Immunoblot second-tier testing employed during the first 4 weeks of disease includes testing of both IgM and IgG antibody reactivity. Due to the likelihood of a false-positive test result for IgM indicating a false active infection in later stage patient samples, IgM immunoblot results are not considered reliable for patients with suspected illness greater than 4 weeks in duration [110].

Two-tier testing has provided an adequate approach for Lyme disease diagnosis but suffers from certain weaknesses including the subjectivity and complexity of immunoblot analysis as well as non-standardized lysate preparations and antigen sources [111]. For these reasons, immunoblot analysis and two-tier results have been shown to vary from laboratory to laboratory based on the test strategy used for detection of host antibodies resulting from the particular kit used [112]. Differences between test results largely reflect the antigen variability across different manufacturers [111]. As detailed above, *B. burgdorferi* antigen expression can vary significantly based on the strain and the conditions used to cultivate the organism. More recently, in an effort to standardize the antigens used between Lyme disease diagnostics it has been suggested that the use of whole cell sonicate as a source of antigens be replaced with the utilization of a combination of recombinant *B. burgdorferi* antigens [109]. Although a single antigen would further simplify a diagnostic test for Lyme disease, no single antigen tested to date has shown success at diagnosing Lyme disease across all stages of the disease [111]. A more recent ELISA that targets the conserved VlsE C6 peptide of *B. burgdorferi* has been developed and proposed as a single-tier test. Although it appears to provide increased sensitivity for early stage disease diagnosis, the antigen shows no increased sensitivity for later-stage disease diagnosis with overall lower specificity observed when compared with two-tier testing [113]. Hence, despite its drawbacks, two-tier testing remains the recommended and current method for laboratory diagnosis of Lyme disease [114].

Additional Methods For Diagnosis Of Lyme Disease In Development

Although two-tier testing remains the mainstay of clinical Lyme disease diagnostics, it is important to emphasize that additional methods have been developed and tested. These methods include both direct detection of the presence of spirochetes and indirect detection by serological methods. Direct methods include microscopic observation of whole spirochetes from patient samples, detection of *B. burgdorferi*-specific antigens, *in vitro* culture of spirochetes from patient samples and polymerase chain reaction amplification (PCR) of spirochete nucleic acid targets [111]. Dark field microscopy is useful for specimens where large numbers of spirochetes are expected but the value of diagnosis by microscopy in the clinical laboratory is limited due to the low organism density in clinical samples and similarity to host tissue structures [55]. Direct detection of *B. burgdorferi* antigens by ELISA and dot blot is rarely used in a clinical setting due to low sensitivity [115] and poor specificity and reproducibility [116]. Culture of *B. burgdorferi* from patient samples is also not routinely used in a clinical setting due to variations in *in vitro* growth medium, long sample incubation periods (minimum of 12 weeks), and most importantly very low sensitivity due to small numbers of spirochetes present in blood during infection [111]. Although it appears to be a promising approach for direct detection of spirochetes, PCR has not been widely accepted as a laboratory diagnostic for Lyme disease due to low sensitivity in blood and cerebral spinal fluid and accidental laboratory contamination of samples with small quantities of target DNA that can lead to potential false-positive results [117]. For these reasons, indirect detection of infection by serology using

ELISA and western blot has remained the method of choice in clinical settings. With inherent limitations to two-tier testing discussed above, there exists the opportunity for improvements to current Lyme disease diagnostics.

Immuno-PCR

Technique Summary

Immuno-PCR (iPCR) as first described by Sano *et al.* in 1992 [118] combines the capability for signal amplification afforded by PCR with the flexibility of an ELISA based approach, which can result in overall improvement of conventional antigen detection methodology. The basic design of the assay depends on the analyte (antigen or antibody) being measured. Early versions of the technique used gel electrophoresis to measure the amount of PCR product generated. This not only limited the range of quantification that could be used for immuno-PCR but was also laborious, had low sensitivity and provided limited applicability for quantitative measurement. With the incorporation of real-time PCR into the existing protocol, the amount of reporter oligonucleotide could be quantified with high sensitivity and accuracy over a wide concentration range providing concise and consistent measurements of antigens within a sample [119]. It was also through elimination of the post-PCR processing steps used for gel electrophoresis that both the total assay time as well as overall risk of laboratory contamination were significantly reduced [119].

Following development and optimization by researchers of the iPCR protocol for sensitive and quantitative detection of proteins in samples, studies

were undertaken to determine the sensitivity of the approach as compared to standard ELISA protocols. Results indicated that typically 100-10,000-fold detection limit improvements were observed in almost all applications [120]. iPCR has been applied for sensitive detection of a variety of targets including viral antigens [121], bacterial antigens [122], prions [123] and bacterial toxins [124]. There has been only a limited number of iPCR studies focused on host generated antibody detection, with successful application to the measurement of mumps-specific IgG in human patient serum [125].

Application of iPCR for Lyme Disease

Although two-tier testing based on ELISA followed by immunoblot is the current accepted method of Lyme disease diagnosis, a better approach would be to increase the sensitivity of the current system through incorporation of PCR signal detection combined with the use of recombinant antigens. The issues posed by the current approach including limited sensitivity, subjectivity of analysis and inconsistency in capture antigens have the potential to be alleviated through the application of iPCR and recombinant *B. burgdorferi in vivo*-expressed antigens. An iPCR-based assay design for indirect detection first requires antigens specific for host generated antibodies to be immobilized to a surface, which can include a microtiter plate, magnetic beads or any suitable vessel. The patient sample is then exposed to the antigen-coated surface and any antibodies present in the sample will bind the antigens immobilized on the surface. A secondary reporter antibody coupled to a DNA oligonucleotide is then added and binds to any human antibodies

that were captured by the surface bound antigens. The DNA reporter oligonucleotide attached to the secondary antibody is then amplified by PCR following extensive washing of the complex to remove any unbound reagents. By determining the cycle number where the exponential phase of amplification is achieved during PCR, the amount of antibody present in the patient sample can be quantitatively determined. iPCR has already demonstrated increased sensitivity for other applications and use of recombinant antigens could provide both standardized reagents as well as provide a potential to combine antigens from multiple species and strains in unique configurations [126]. Beyond just sensitivity, the multiplex capabilities, objective analysis and ease-of-use of iPCR [127] make it a strong candidate for development of a new Lyme disease diagnostic. The workflow for iPCR could also be further simplified and automated by transfer to automated systems and even microfluidic point-of-care diagnostic platforms as has been accomplished for similar protocols [128]. Reduction in the assay complexity would provide the capability for more routine, affordable and high-throughput diagnostic testing of Lyme disease patients.

The overall goals of the research detailed in this dissertation were to 1) develop an iPCR protocol suitable for detection of a *B. burgdorferi* infection, 2) apply the protocol using *in vitro* expressed recombinant antigens for direct detection of spirochetes and indirect detection of host generated antibodies in an infected murine model and 3) determine the level of sensitivity and specificity of the optimized protocol with more comprehensive testing of human Lyme disease patient and healthy donor samples.

CHAPTER TWO:
ENHANCED DETECTION OF HOST RESPONSE ANTIBODIES TO *BORRELIA BURGDORFERI* USING IMMUNO-PCR

Preface

The first complete draft of the chapter was written by MDH. Comments from MWJ and reviewers were incorporated into the final version presented here. This chapter was published previously and is reprinted here with permission. Halpern MD, Jain S, Jewett MW. 2013. Enhanced detection of host response antibodies to *Borrelia burgdorferi* using immuno-PCR. Clin Vaccine Immunol. 20(3):350. DOI: 10.1128/CVI.00630-12.

Introduction

Lyme disease is the leading vector-borne bacterial disease in the world with approximately 30,000 cases reported in the United States alone each year [37]. Lyme disease has been characterized as the fastest growing zoonotic disease in North America. According to the Centers for Disease Control and Prevention (CDC), the number of clinical cases of Lyme disease has more than doubled over the past 10 years making this emerging infectious disease a major public health concern [37]. Accurate diagnosis is currently the greatest challenge for the clinical management of Lyme disease. Misdiagnosis is common as the clinical manifestations of the disease are not unique and detection of a *B. burgdorferi* infection is difficult and prone to misinterpretation [111, 129]. Different approaches for laboratory testing, such as microscopy, genomic DNA amplification and serology have been examined

with currently accepted laboratory diagnostics relying primarily on detection of a serological response to *B. burgdorferi* antigens [111, 130, 131].

Current methods for detection of Lyme disease in a clinical setting as approved by the CDC entail a two-tiered approach using a first tier enzyme immunoassay (EIA) followed by a second tier immunoblot for both IgM and IgG *B. burgdorferi* specific antibodies using whole cell *B. burgdorferi* lysates, recombinant antigens or various combinations depending on the commercial kit used [111]. Although adequate, the approach suffers from certain drawbacks including the subjectivity of immunoblot analysis and the lack of standardization of antigen source and lysate preparations. These challenges have resulted in discordant results between test strategies for detection of host antibodies based on the kit used [112] largely due to lysate/antigen reagent variability [111]. The most effective approach appears to be the use of a combination of recombinant antigens to replace whole organism sonicates as no single antigen has been found to be sufficient for accurate diagnosis [111].

Other methods for detection of Lyme disease include live culture and approaches employing polymerase chain reaction (PCR). Live culture has shown limited success in a clinical setting, is time consuming and requires complex media that have a limited commercial supply [111]. PCR appears to be the most promising method for direct detection of spirochetes but has not been widely accepted for laboratory diagnosis due to low sensitivity in cerebral spinal fluid and blood and the potential false-positive results due to accidental laboratory contamination of samples with small quantities of target

DNA [117]. An improved approach would be to utilize the sensitivity of PCR combined with an antigen based detection system that is much less susceptible to false positive results.

Immuno-PCR (iPCR) was first introduced by Sano *et al.* in 1992 [118] and combines the amplification power of PCR with the versatility of EIA resulting in improved conventional antigen detection sensitivity. Using iPCR, a typical 100-10,000-fold improvement over the detection limit of the EIA has been obtained in almost all applications [120]. iPCR has been used to detect viral antigens [121], bacterial antigens [122], prions [123] and bacterial toxins [124]. There has also been a limited application of iPCR for antibody detection, such as the measurement of mumps-specific immunoglobulin G in human serum [125].

The combination of an iPCR approach and recombinant *B. burgdorferi in vivo*-expressed antigens has the potential to alleviate a number of the issues posed by Lyme disease diagnostics. Recombinant antigens not only have the potential to standardize the reagents used for Lyme disease diagnostics but also provide the opportunity to combine antigens from multiple strains/species. The sensitivity, ease-of-use, objective analysis and multiplex capabilities of iPCR [127] also makes it an ideal platform for Lyme disease detection. Furthermore, iPCR has the ability to be translated to an automated point-of-care diagnostic platform using microfluidics [128] that may allow routine, high-throughput and affordable diagnostic testing of Lyme disease patients. The goal of this research was to explore the initial application of iPCR using recombinant antigens for detection of either host generated

antibodies or direct detection of spirochetes in *B. burgdorferi* infected samples.

Materials and Methods

Bacterial Strains

B. burgdorferi clone B31 A3 [132] and B31 A34/pBSV2G-*loxP-flaB*p-*gfp* [133] were used in these studies. Spirochetes were grown in liquid Barbour-Stoenner-Kelly (BSK) II medium supplemented with gelatin and 6% rabbit serum [134] and plated in solid BSK medium as previously described [135]. All spirochete cultures were grown at 35°C and supplemented with 2.5% CO_2. Gentamicin was used at 40 μg/ml. *Escherichia coli* strains DH5α and BL21 (Novagen, Billerica, MA) were grown in LB broth, on LB agar plates or in Magic Media (Invitrogen, Carlsbad, CA) containing 100 μg/ml ampicillin.

Mouse Infections

The University of Central Florida (UCF) is accredited by the International Association of Assessment and Accreditation of Laboratory Animal Care. Protocols for all animal experiments were prepared according to the guidelines of the National Institutes of Health and approved by UCF's Institutional Animal Care and Use Committee. For the serological detection experiments, the hair on the upper backs of three mice (C3H/HeN, 6- to 8-week old females; Harlan Laboratories, Inc, Dublin, VA) was removed by shaving and the mice were needle inoculated intradermally on the upper back

with *B. burgdorferi* strain B31 A3 at a dose of $1x10^5$ spirochetes divided between two 50 μl inoculations. The number of spirochetes inoculated into mice was determined using a Petroff-Hausser counting chamber and verified by colony forming units (cfu) counts in solid BSK medium. Total plasmid content of each inoculum was confirmed to be as expected [136]. Whole blood samples were collected from the three inoculated mice as well as one non-inoculated mouse by submandibular bleed pre-inoculation and at days 1, 3, 4, 7, 9, 11, 14, 16, 18 and 21 post-infection. The coagulated blood was spun at 4,000xg for 9 minutes to prepare serum. For the spirochete detection experiments, six mice (C3H/HeN, 6-8 week old females, Harlan Laboratories, Inc, Dublin, VA) were inoculated intradermally with *B. burgdorferi* strain B31 A3 at a dose of $1x10^5$ spirochetes. Approximately 50 μl of blood were collected by submandibular bleed from all mice prior to inoculation. Subsequently, to prevent complications due to oversampling, approximately 50 μl of blood/mouse were collected every day from groups of two mice so that each group of two mice was bled every three days over a time period of 14 days. All blood samples (pre- and post-inoculation) were supplemented with an equal volume of 0.5M sodium EDTA to prevent coagulation.
Similar to plating of *in vitro* grown *B. burgdorferi,* 50 μl of blood from each mouse was combined with BSK plating medium [135] supplemented with a *Borrelia* antibiotic cocktail consisting of 20 μl /ml phosphomycin (MP Biomedicals, Santa Ana, CA), 50 μl /ml rifampicin (Fisher Scientific, Waltham, MA) and 2.5 μl /ml amphotericin B (Fisher Scientific, Waltham, MA), all

solubilized in 20% DMSO, poured into sterile petri plates, allowed to solidify and incubated as indicated above for approximately 7 days until *B. burgdorferi* colonies were visible in the solid medium.

Immunoblot and C6 ELISA

Total *B. burgdorferi* lysate for immunoblot analysis was prepared from a 500 ml culture of $1x10^8$/ml *B. burgdorferi* B31 A3. Spirochetes were harvested by centrifugation and washed two times in 30 ml phosphate buffered saline, pH 7.4 (PBS). Washed cells were resuspended in 30 ml PBS and disrupted by sonication on ice using a Misonix model S-4000 sonciator at 40% amplitude for four repetitions at 20 seconds each. Total protein in the sonicate was normalized to 1 mg/ml with PBS based on absorbance at 280 nm and 75 μg of protein were separated by 12.5% polyacrylamide gel electrophoresis. Following protein transfer, nitrocellulose membranes were incubated for 1 hour with pre- and post- inoculation mouse sera diluted 1:200 in Tris buffered saline/0.05% tween, pH 7.6 (TBST), washed twice with TBST, incubated with HRP-conjugated goat anti-mouse IgG/IgM (Chemicon International, Billerica, MA) for 1 hour, washed twice with TBST and the signal was detected using the SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Scientific, Rockford, IL). The C6 *B. burgdorferi* ELISA was performed according to manufacturer's protocol (Immunetics, Boston, MA) with the exception of the use of HRP-conjugated goat anti-mouse IgG/IgM secondary (Chemicon International, Billerica, MA) at a 1:5000 dilution in place

of the anti-human reporter antibody provided with the kit when mouse sera were analyzed.

Cloning and Expression of Recombinant GST Tagged Antigens

In frame glutathione-S-transferase (GST) fusion proteins for OspC, BmpA and the VlsE C6 peptide were generated by PCR amplifying the corresponding coding regions without the signal sequences from *B. burgdorferi* genomic DNA using primer pairs P1 and P2 (OspC), P3 and P4 (BmpA) or P5 and P6 (VlsE C6) engineered with BamHI or SalI restriction sites (Table 1) and Phusion polymerase (New England Biolabs, Ipswich, MA). PCR products were purified (Qiagen, Valencia, CA), digested with restriction enzymes (New England Biolabs, Ipswich, MA) and cloned into BamHI/SalI-digested pGEX-6P-1 (GE Healthcare, Piscataway, NJ) to generate translational fusions with GST at the N-terminus. Subsequent clones were selected and sequence confirmed by dideoxy sequencing. Hemagglutinin (OspC) and C-Myc (BmpA) tags were included at the C-terminus for determination of protein purity by immunoblot. pGEX-6P-1 plasmids carrying *ospC*, *bmpA* or *vlsE c6* were transformed into a BL21 strain of *E. coli* (Novagen, Billerica, MA). Protein expression and purification were performed according to the procedures outlined in the Bulk GST Purification Module (GE Health Sciences, Piscataway, NJ).

Table 1. iPCR DNA oligonucleotide sequences used in this study

Oligo number	Oligo ID	Sequence (5'-3')[a]
T1	Template 1 (IgG coupled)	BIOTIN-agcctcagaccaagccagacaactgcctcgtgacgttgctgcccctaccaacgtacccctacgagtcc
T1F	Template 1 Forward	agcctcagaccaagccagac
T1R	Template 1 Reverse	ggactcgtaggggtacgttgg
T1P	Template 1 Probe	FAM-actgcctcgtgacgttgctgcccct-BHQ1
T2	Template 2 (IgM coupled)	BIOTIN-aggaggagggtcaagtcaccaacgctgctccaggccatcgtgctgatctggaccctggatcgagtga
T2F	Template 2 Forward	aggaggagggtcaagtcacc
T2R	Template 2 Reverse	tcactcgatccagggtccag
T2P	Template 2 Probe	MAX-acgctgctccaggccatcgtgctga-BHQ1
P1	OspC partial HA F	CGGGATCCCATATGtgtaataattcagggaaagatgg
P2	OspC HA R	ACGCGTCGACttaCGCATAATCCGGCACATCATACGGATAaggttttttggactttctgc
P3	BmpA partial myc F	CGGGATCCCATatgtgtagtggtaaaggtagtcttg
P4	BmpA myc R	ACGCGTCGACttaCAGATCTTCTTCAGAAATAAGTTTTTGTTCaataaattctttaagaaacttctcataac
P5	C6 Bb F	CGGGATCCCATatgaagaaggatgatcagattg
P6	C6 Bb R	ACGCGTCGACttacttcacagcaaactttccatc

[a] Uppercase letters indicate non-template sequence used for addition of terminal restriction sites and/or epitope tags. BHQ1, black hole quencher 1.

iPCR Reagent Preparation

iPCR assays were assembled as two-sided (sandwich) as detailed in Figure 4 for both host antibody (A and B) and spirochete capture (C). Whole cell lysate used for immunoblot analysis (preparation described above) and GST-fusion recombinant antigens were used to coat magnetic beads for host antibody capture using Dynabeads Antibody Coupling Kit (Invitrogen, Carlsbad, CA). Bead coupling reactions were performed overnight according to the manufacturer's protocol using 20-30 μg antigen(s) per mg Dynabeads M-270 Epoxy. The primary antibody used for spirochete capture consisted of protein A purified anti-*B. burgdorferi* polyclonal antibody raised in rabbits against whole cell preparations of *B. burgdorferi* clone B31 ATCC #35210 (Acris Antibodies, San Diego, CA) and was coupled to magnetic beads as described above. Protein coated beads were stored at 4°C. The streptavidin conjugated reporter antibodies were prepared using the Lightning-Link Streptavidin Conjugation Kit (Innova Biosciences, Cambridge, United Kingdom) using polyclonal anti-*B. burgdorferi* (Acris Antibodies, San Diego, CA), goat anti-mouse IgM/IgG (Sigma-Aldrich, St. Louis, MO), goat anti-human IgG (Invitrogen,

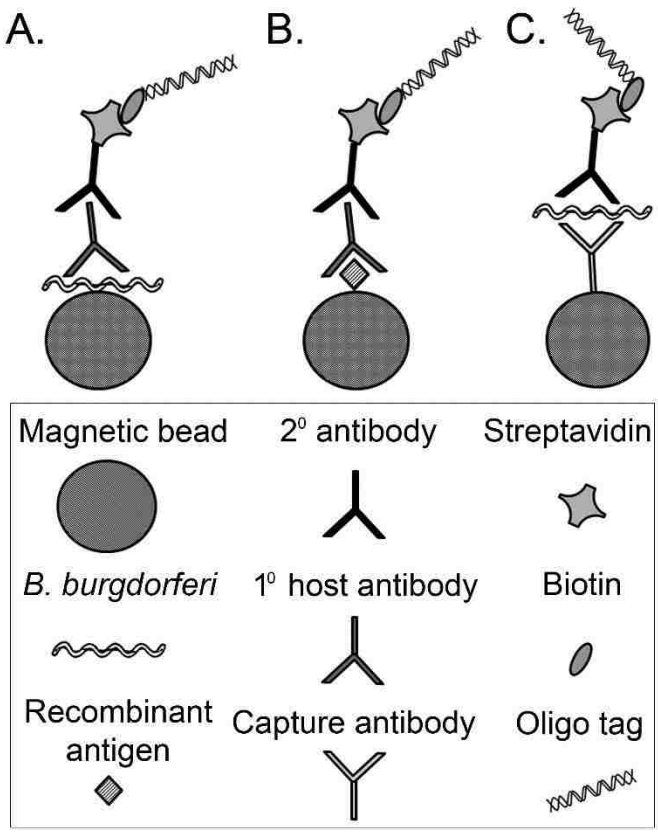


Figure 4. Schematic representation of iPCR assay for detection of Lyme disease biomarkers. (A) Intact spirochete or (B) recombinant protein antigen coupled to magnetic beads was used to capture B. *burgdorferi*-specific host generated antibodies. A biotinylated DNA oligonucleotide reporter molecule coupled to a streptavidin conjugated reporter antibody was amplified by qPCR for detection and quantification. (C) Anti-B. *burgdorferi* antibody coupled to magnetic beads was used for spirochete capture with detection accomplished by qPCR amplification of the DNA oligonucleotide coupled reporter antibody similar to detection of host antibody.

Carlsbad, CA) or goat-anti-human IgM (Invitrogen, Carlsbad, CA) according to manufacturer's protocols using an overnight incubation. Following conjugation, 10 μl of streptavidin labeled antibody was diluted 1:50 in TBST and 100 nM of single stranded biotin-labeled oligonucleotide template was added and the mixture rotated at room temperature for 30 minutes for antibody-oligo conjugation. Oligonucleotide sequences T1 (IgG

coupled) and T2 (IgM coupled) used for tagging are listed in Table 1. The oligonucleotide linked streptavidin conjugated antibody was then diluted to a 1:100 working stock (1:5000 final dilution) and stored at 4°C.

iPCR assay

Following reagent preparation, 10 μl of antigen or antibody coated magnetic beads were incubated in 500 μl TBST for 30 minutes at 25°C on a rotator. Following preliminary washing, beads were resuspended in 500 μl TBST and 5 μl serum (mouse or human), 10 μl spirochetes suspended in HN buffer or blood ($1x10^8$-$1x10^4$/ml *B. burgdorferi* B31 A3) or no serum/spirochete (negative control) and incubated at 25°C rotating for 30 minutes. Beads were subsequently washed and resuspended in 300 μl TBST with the addition of 100 μl each of IgG and IgM diluted (1:5000) biotinylated oligonucleotide streptavidin coupled reporter antibody (anti-mouse IgM/IgG, anti-human IgG, anti-human IgM or anti-*B. burgdorferi*) and incubated at 25°C rotating for 30 minutes. Following assembly of the immune complex, beads were washed three times with 900 μl TBST followed by magnetic bead capture. Washed immune complex coupled beads were resuspended in 20 μl TBST for subsequent PCR amplification.

Signal Amplification by Real-Time PCR

To amplify the signal of the immune complex, real-time PCR was performed using an Applied Biosystems 7900 HT (Life Technologies, Grand Island, NY) and IQ Supermix (Bio-Rad, Hercules, CA) supplemented with

synthetic primers and probes T1F/T1R/T1P (IgG detection) or T2F/T2R/T2P (IgM detection) (Table 1). Duplicate reactions were prepared in 20 μl volumes containing 5 μl of iPCR assay processed beads as template, 10 μl of 2X reaction mix, 0.2 μM each primer and 0.4 μM fluorophore labeled probe. Cycle parameters included a preliminary denaturation (95°C, 20 sec), followed by 40 cycles of denaturation (95°C, 1 sec) and annealing/extension (60°C, 20 sec). The fluorescent signal was collected at the FAM wavelength for IgG reactions and MAX wavelength for IgM reactions. The quantification cycle (Cq) for each reaction was determined using automatic baseline and threshold settings. The average and standard deviation for uninfected/healthy samples were used to determine the background level of amplification as is commonly observed for iPCR protocols. Positive threshold values were established at three times the standard deviation for background levels.

Human Sera

Retrospective, de-identified human Lyme disease and healthy control serum samples were kindly supplied by Dr. Martin Schriefer (Centers for Disease Control and Prevention, Fort Collins, CO). Patient sera were collected from 18 Lyme disease patients from endemic Lyme disease regions upon initial visit to a physician and 10 days post-initial visit (n = 36). According to the CDC's 2-tiered serological analysis of the samples, 5 of the patients were 2-tiered positive at both the initial and follow-up time points, 3 of the patients were 2-tiered negative a both time points and 10 of the patients were 2-tiered negative at the initial visit but 2-tiered positive 10 days later. Human

control samples consisted of sera collected from healthy blood donors living in non-Lyme endemic areas (*n* = 5).

Statistical Analysis

Spearman rank correlation analyses were performed using GraphPad Prism, version 5.0.

Results

iPCR Using Intact Spirochetes Provided Earlier Detection of Host Response Compared to Immunoblot and C6 ELISA in a Murine Model

The general approach for detection of a host antibody immune response by immunoassay is to use sonicated or otherwise disrupted organisms to generate protein antigens for antibody capture and subsequent detection. However, we hypothesized that this approach may have limited success for effectively capturing anti-*B. burgdorferi* antibodies in experimentally infected mouse sera as the majority of the *B. burgdorferi* proteins in the total cell lysate are not likely to be immunogenic. Although *B. burgdorferi* lysate is known to harbor antigenic proteins recognized by mouse and human immune sera, these proteins represent a small percentage of the total proteins in the lysate and therefore may not provide improved sensitivity of detection of an immune response to *B. burgdorferi* infection. In an effort to develop a sensitive, objective method for detection of host antibodies against *B. burgdorferi* antigens, magnetic beads were coated with a polyclonal anti-*B. burgdorferi* Antibody in order to capture formalin fixed intact spirochetes, resulting in the generation of magnetic beads coated with intact spirochetes

(Figure 5). We predicted that this strategy would result in magnetic beads coated in an enriched pool of spirochete antigenic outer surface proteins capable of interacting specifically with host antibodies produced in response to a *B. burgdorferi* infection. The sensitivity of iPCR using intact spirochetes to capture host antibodies was compared to pre-existing diagnostic methods including a commercial C6 ELISA and immunoblot using an *in vivo* murine model. iPCR resulted in the earliest objective detection of a positive infection on day 11 post-inoculation (Figure 6A). In comparison, C6 ELISA and immunoblot exhibited positive detection of anti-*B. burgdorferi* antibodies at day 14 and day 21 post-inoculation, respectively (Figure 6B and 6C). The approximate molecular weights of the immunodominant proteins detected on the immunoblot included 18 kilodaltons (kDa), 23 kDa, 33 kDa, 39 kDa and 66 kDa, which are consistent with the sizes of the bands typically present on a Lyme disease diagnostic immunoblot [99, 110]. Uninfected mouse serum was negative by all three methods at all time points tested (Figure 6).

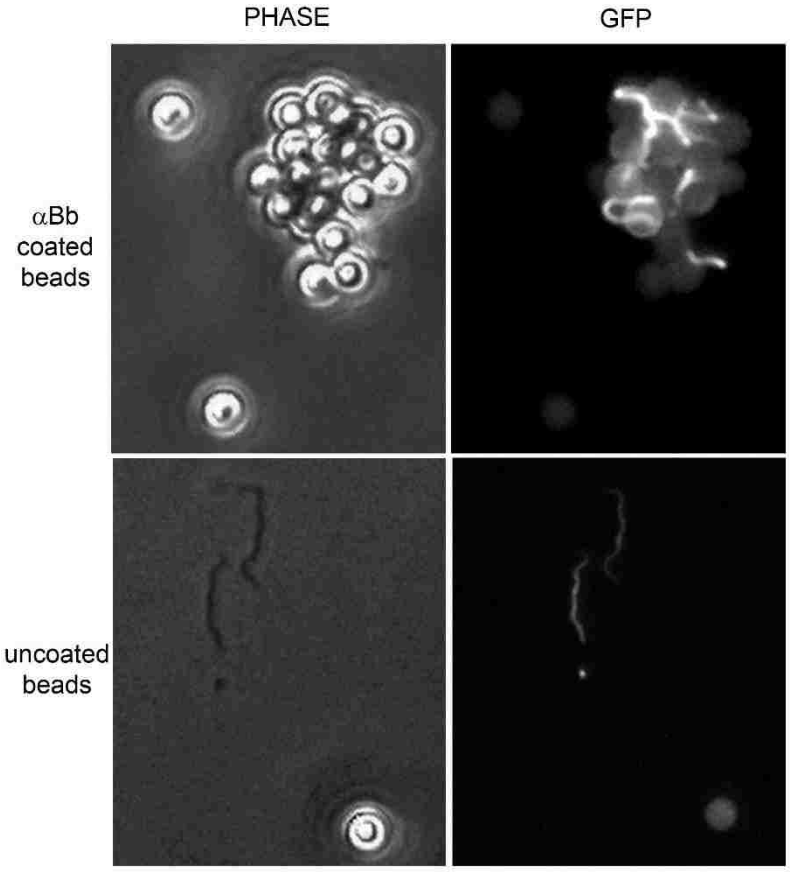


Figure 5. B. *burgdorferi* captured on magnetic beads provides a reagent for host antibody detection by iPCR. Phase contrast and fluorescent microscopy at 510 nm (GFP) and 400x magnification (400x) was used to determine capture of formalin fixed B. *burgdorferi* expressing green fluorescent protein on beads coated with anti-B. *burgdorferi* polyclonal antibodies (top panels) or uncoated beads (bottom panels).

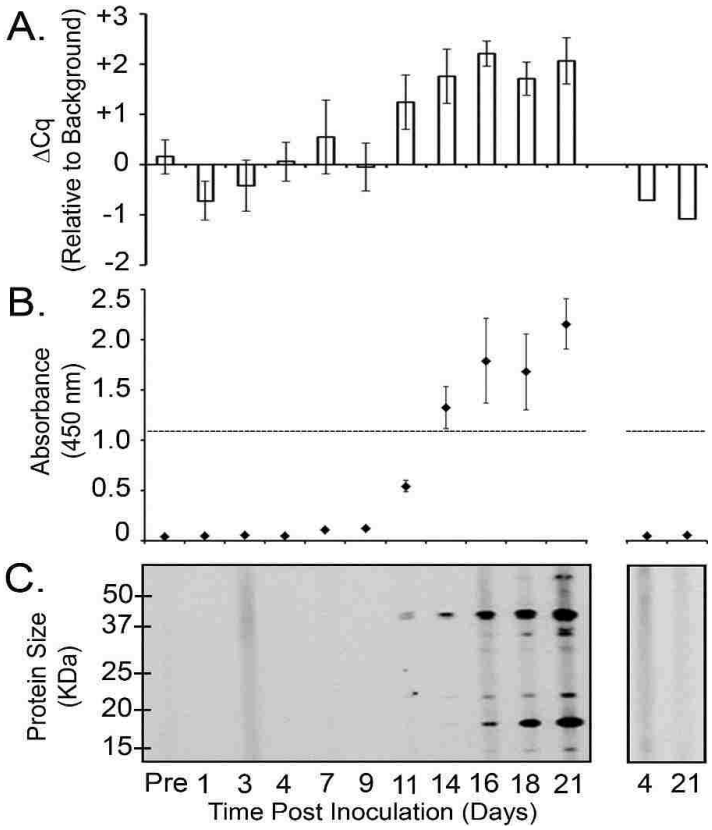


Figure 6. iPCR demonstrated earlier detection of host response antibodies in B. *burgdorferi* infected mice compared to C6 ELISA and immunoblot. Mouse sera were collected prior to inoculation (pre), at specific days post-intradermal inoculation with $1x10^5$ B. *burgdorferi* B31 A3 (left panels), or from uninfected mice (right panels) over the course of 21 days. (A) Undiluted sera were analyzed for detection of B. *burgdorferi* IgG antibodies using iPCR. Closed system, real time PCR of the DNA reporter molecule was performed using a Taqman-based fluorescent probe assay. The mean quantification cycle (Cq) background signal, determined using uninfected sera plus three standard deviations was designated as the call threshold for a positive detection event and indicated here as $\Delta Cq = 0$. Data are shown as the Cq value for each sample minus the mean background Cq plus three standard deviations (ΔCq). Each data point represents the average of three mice and the standard deviation between samples is shown. (B) C6 ELISA (Immunetics, Inc., Boston, MA) was performed according to the manufacturer's instructions with the exception that the secondary antibody was peroxidase-conjugated goat anti-mouse IgM/IgG (1:5000). The threshold absorbance for the test is indicated (horizontal broken line). Each point represents the average of three mice and the standard deviation between samples is shown. (C) Total B. *burgdorferi* sonicate was separated by SDS-PAGE and analyzed by IgM/IgG immunoblot using immune and pre-immune mouse sera diluted 1:200. The positions of the protein standards depict molecular weights in kilodaltons (kDa). Data are representative of three mice analyzed.

iPCR Using Recombinant GST-OspC and GST-BmpA Provided Improved Sensitivity of Detection of Murine Host Antibodies

Although beads coated with intact *in vitro* grown spirochetes provided early detection of anti-*B. burgdorferi* antibodies as compared to the C6 ELISA and immunoblot (Figure 6), we hypothesized that specific recombinant antigens known to be actively expressed during murine infection could potentially result in a more sensitive approach. Known *B. burgdorferi in vivo*-expressed antigens OspC and BmpA [111] were produced and purified as recombinant N-terminal GST-tagged fusion proteins in *E. coli*. Magnetic beads coated with either recombinant protein were used to capture host antibodies generated against OspC or BmpA, respectively, and IgM and IgG antibodies against each protein were individually quantitated using our iPCR assay. GST-OspC coated beads resulted in a marked increase in detection of host antibodies starting at day 7 post inoculation for both IgG and IgM (Figure 7A) with a gradual decrease in IgM back to baseline by day 21 and a minimal decrease in IgG signal to the same time point. GST-OspC-coated beads provided a dramatic increase in the level of IgG detection (ΔCq = 10) as compared to the level of iPCR detection of host antibodies using intact spirochete-coated beads (ΔCq = 2.5). GST-BmpA-coated beads provided robust positive detection of IgG antibodies beginning at day 9 followed by a minimal decrease in the detection signal out to day 21 (Figure 7B). IgM antibodies directed against BmpA demonstrated a slight increase in signal over the 21-day time course of infection but were not significantly detected above background, suggesting that BmpA does not elicit a serodiagnostic IgM

response. Together these data suggest that the use of magnetic beads coated with specific recombinant *B. burgdorferi in vivo*-expressed antigens results in robust iPCR detection of a humoral response in mice experimentally infected with *B. burgdorferi* and development of an iPCR assay that quantitates the host response to multiple *B. burgdorferi* antigens may result in an improved diagnostic method.

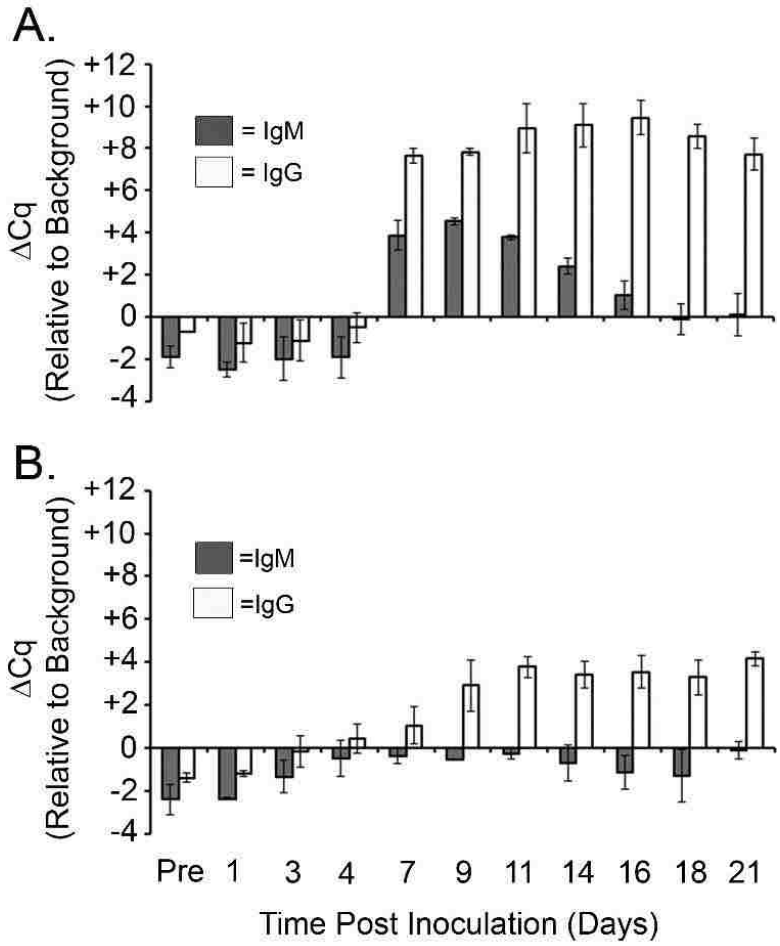


Figure 7. iPCR using recombinant antigens OspC and BmpA provided enhanced detection sensitivity for both IgG and IgM isotypes in a murine model of infection. Magnetic beads coated with either purified recombinant GST-OspC (A) or GST-BmpA (B) protein were used to capture host response antibodies from pre-immune (pre) or post-immune mouse sera collected over a time period of 21 days. IgM- and IgG-specific reporter antibody-DNA conjugates detected anti-B. *burgdorferi* antibodies captured by each set of antigen coated beads. The IgM (gray bars) and IgG (white bars) response to each antigen was determined for each mouse by multiplex quantitative PCR using distinct probes specific for the IgM- and IgG-specific DNA reporters molecules. The mean quantification cycle (Cq) background signal, determined using uninfected sera plus three standard deviations was designated as the call threshold for a positive detection event and indicated here as $\Delta Cq = 0$. Data are shown as the Cq value for each sample minus the mean background Cq plus three standard deviations (ΔCq). Each data point represents the average of two mice and the standard deviation between samples is shown.

iPCR Demonstrated a Strong Correlation with a Commercial ELISA for Detection of Host Antibodies in Human Serum Using the VlsE C6 Peptide

As recommended by the CDC, the first step of two-tier testing for Lyme disease is the use of a sensitive enzyme immunoassay. Although a number of commercial kits exist for testing, the C6 peptide of the VlsE locus has been shown to be a sensitive and effective predictor for follow-up testing by immunoblot and is available as a commercial testing kit. In order to directly compare the ability of our iPCR assay to detect human antibodies produced against the VlsE C6 peptide with that of an FDA-approved C6 antibody detection method, a panel of human serum samples that consisted of samples from 18 individuals collected at both an initial visit to the clinic and a ten day follow up appointment (n = 36) along with sera collected from 5 healthy patients from non-Lyme endemic areas were analyzed by iPCR and using the C6 Lyme ELISA (Immunetics, Inc., Boston, MA). iPCR detection of C6-specific host antibodies demonstrated a strong correlation with that of the commercial C6 ELISA (r_s = 0. 895, P < 0.0001) (Figure 8). The iPCR assay differed from the C6 ELISA in that the iPCR assay provided a separate measurement of C6 IgM and C6 IgG antibodies as opposed to the C6 ELISA, which quantitated a combined value for both C6 IgM and C6 IgG antibodies. Therefore, the iPCR result was considered positive if C6 IgM and/or C6 IgG antibodies were detected at or above the established call threshold. All 21 samples that demonstrated a positive result by the C6 ELISA were also positive according to C6 iPCR (Figure 8). Of the four samples determined to be equivocal by the C6 ELISA, three of the sera were found to be negative by

C6 iPCR; whereas, one sample tested positive for IgM using this method. Furthermore, of the 11 serum samples that tested negative by C6 ELISA, five of those sera resulted in positive detection of IgM by C6 iPCR. Of note, all iPCR positive samples in this group had ΔCq values of 1 or below. All serum samples collected from known healthy individuals tested negative by both C6 ELISA and C6 iPCR. Together these results suggested that iPCR may have improved ability to detect host antibodies to the VlsE C6 peptide compared to a current commercial method.

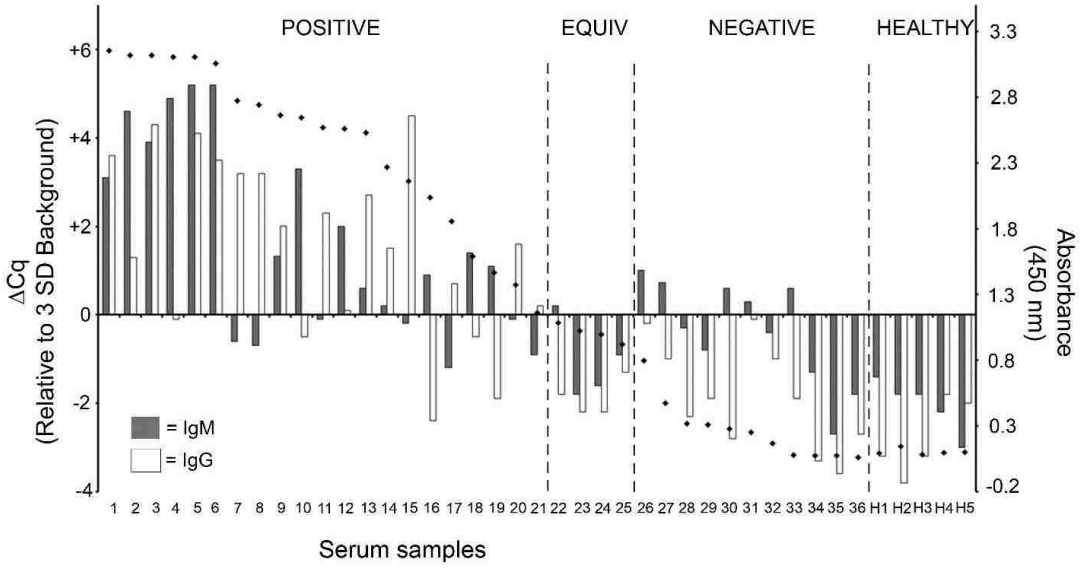


Figure 8. Recombinant antigen iPCR successfully quantified *B. burgdorferi* VlsE C6 peptide antibodies in human serum samples. Results for 36 serum samples from 18 Lyme disease patients collected upon initial visit to a clinic and at a 10 day follow up visit and 5 healthy controls using a multiplex iPCR protocol to quantitate both IgM (gray bars) and IgG (white bars) isotypes using recombinant *B. burgdorferi* VlsE C6 peptide coated magnetic beads. A call threshold (ΔCq = 0) was assigned at greater than or equal to three standard deviations above the mean background signal determined using serum from healthy individuals. Serum samples were also tested using a commercial C6 ELISA (Immunetics, Boston, MA) (diamonds), which was performed according to manufacturer protocol with a call threshold for an absorbance (450 nm) of 1.1 used according to the manufacturer's protocol. The C6 ELISA value represents combined measurement of C6 IgM and IgG antibodies. The patient data (1-36) are grouped into three categories: positive, equivocal (equiv) and negative according to the C6 ELISA values. Samples H1-H5 correspond to the sera collected from the healthy controls and are grouped accordingly (healthy). The calculated Spearman rank correlation (r_s) was 0.734 ($P < 0.0001$) for C6 iPCR IgM versus C6, 0.826 ($P < 0.0001$) for C6 iPCR IgG versus C6, and 0.895 ($P < 0.0001$) for C6 iPCR IgM and/or IgG versus C6.

iPCR Directly Detected *B. burgdorferi* in Blood

The demonstrated power of iPCR to detect ultra-low protein levels [120] suggests that this method may be a promising tool for direct detection of *B. burgdorferi* in clinical samples. iPCR was shown to be successful for capture of live *B. burgdorferi* using magnetic beads coated with polyclonal anti-*B. burgdorferi* antibodies (Figure 5). This finding suggested the potential for iPCR to directly quantitate spirochetes from within patient samples. To test the sensitivity of iPCR detection of spirochetes, *in vitro* grown *B. burgdorferi* were serially diluted in HN buffer (10^6-10^2 spirochetes). iPCR detection of spirochetes demonstrated a robust dilution curve and a level of detection of less than 1,000 organisms (Figure 9A). Detection of *in vitro* grown *B. burgdorferi* spiked into whole uninfected mouse blood resulted in a ten-fold lower limit of detection of 10,000 spirochetes (data not shown), suggesting that components of the blood may have an inhibitory effect on the function of the iPCR assay. To correlate the sensitivity of iPCR detection of spirochetes in blood with quantitation of the number of spirochetes present in the blood of infected mice, cohorts of mice were infected with $1x10^5$ *B. burgdorferi* B31 A3 and blood samples collected every 24 hours for a period of fourteen days. The number of spirochetes/ml of blood, as determined by cfu counts on solid medium, were found to increase over the first week of infection and reached a peak number of approximately 2,500 spirochetes/ml of blood on day 8 post-inoculation (Figure 9B). The *B. burgdorferi* colonies that grew out of the infected blood within the solid BSK medium demonstrated morphology and growth pattern similar to what is typically observed for spirochete colonies

derived from *in vitro* grown cultures (data not shown). Together, these data suggest that although iPCR is a promising method for direct detection of spirochetes in *B. burgdorferi* infected samples, the sensitivity of the method is currently below the required level of detection.

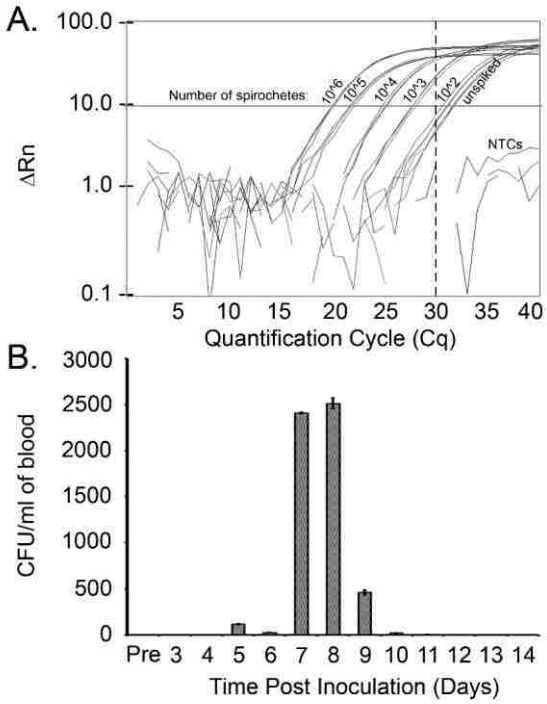


Figure 9. iPCR has the potential to directly detect B. *burgdorferi* in infected samples. (A) Live spirochetes were serially diluted in HN buffer (106-102 spirochetes) and tested in triplicate using iPCR to detect organism capture using anti-*B. burgdorferi* antibody coated magnetic beads. A call threshold was assigned at greater than or equal to five times the standard deviation (Cq = 30, vertical broken line) above the mean background signal, as determined using HN buffer alone (unspiked). PCR non-template controls (NTCs) included water and TBST used during the iPCR protocol. (B) Six mice were prebled (pre) and inoculated intradermally with $1x10^5$ *B. burgdorferi* strain B31 A3. Approximately 50 µl of blood/mouse was collected every day from groups of two mice every three days over a time period of 14 days. Blood collected from each mouse was plated in solid medium using 50 µl of blood and supplemented with a *Borrelia* antibiotic cocktail (see Materials and Methods for details) and the number of colony forming units (cfus) per ml of blood determined. Data shown are the average cfus/ml for the two mice sampled at each time point.

Discussion

There is a critical need for development of innovative methods for improved diagnosis of Lyme disease. Because of its immunological specificity, signal amplification power and potential for high-throughput automation, iPCR is a strong candidate for development of a robust method to overcome the challenges of Lyme diagnosis. We have demonstrated the first application of iPCR for detection of host antibodies against *B. burgdorferi* in both a murine model and human sera.

iPCR Using Recombinant *B. Burgdorferi in Vivo*-Expressed Antigens Is a Sensitive Method for Detection of Host Response Antibodies in Infected Mice

An iPCR assay that incorporated attachment of intact spirochetes to magnetic beads provided approximately equivalent sensitivity to current diagnostic methods including C6 ELISA and immunoblot when tested in a murine model. However, it is well known that *B. burgdorferi* can alter its surface protein expression based on its environment [137-139]. These data have led to the conclusion that *in vitro* grown spirochetes likely do not present equivalent amounts and types of surface proteins as would be encountered by the host immune system in an active *B. burgdorferi* infection and suggest that the use of multiple *in vivo*-expressed recombinant antigens may improve assay sensitivity [140].

B. burgdorferi has been shown to express a number of antigens during an active infection that can be utilized as recombinant antigens including OspC [141], BmpA [142] to detect host antibodies against *B. burgdorferi* [99].

We hypothesized that saturating the magnetic beads with recombinant *in vivo*-expressed antigenic proteins would provide more binding targets and hence higher sensitivity than intact spirochetes. This was evident in the fact that active infection was detected on day 7-9 post-inoculation using recombinant antigen coated beads as compared to day 11 using intact spirochete coated beads and with a stronger signal above background, ΔCq =5-10 compared to ΔCq = 2.5, respectively. This approach also provides the opportunity to utilize multiple specific antigens either in a combined or individual assay that can be objectively quantified by qPCR.

Recombinant Antigen iPCR Successfully Quantified *B. burgdorferi* VlsE C6 Peptide Antibodies in Human Serum Samples

The immunodominant C6 peptide domain of the VlsE protein has proven successful as a diagnostic antigen [143] and has become a popular choice for first-tier testing prior to follow-up immunoblot testing [144]. An iPCR assay employing a recombinant C6 peptide was developed and compared to an existing commercial kit that uses the same antigen. iPCR detection of C6 antibodies in human sera demonstrated a strong correlation with that of the commercial C6 Lyme ELISA. The C6 ELISA assay results in a combined score for detection of both IgG and IgM isotypes. To provide an additional level of discrimination, the iPCR protocol separately quantitates IgG and IgM antibodies using distinct qPCR template tags and fluorophores, resulting in an individual IgG and IgM iPCR score for each serum sample. All C6 ELISA positive sera were found to be positive for IgG and/or IgM C6 antibodies by iPCR. The added ability of the iPCR assay to differentially quantitate antibody

isotypes for a specific antigen of interest in a single sample may provide important information regarding the disease stage at the time of testing, as IgM is typically produced early in infection with IgG produced later and for longer durations [99, 109].

Of the serum samples that were found to be equivocal or negative by C6 ELISA, a subset of samples in each category was found to be positive by the C6 iPCR assay. These results imply that the iPCR assay may have increased sensitivity of detection over the C6 ELISA; however, further analysis of a larger serum panel is required to fully support this finding. Serum samples from “healthy” individuals with no known exposure to *B. burgdorferi* tested negative by both C6 ELISA and iPCR, suggesting equivalent specificity for the two methods. However, considering the small sample size (n = 5), additional samples need to be tested to confirm this result.

iPCR Has the Potential for Direct Detection of Spirochetes in Infected Samples

In an effort to test applicability of iPCR for direct detection of spirochetes within a sample, it was determined that 1,000 spirochetes were needed in buffer and 10,000 organisms where needed in blood. In the murine model used for development of the protocol, the maximum spirochete load in blood was measured to be approximately 2,500 spirochetes/ml. Therefore the current protocol is unable to directly detect spirochetes during an active murine infection. It has been estimated that the average number of cultivable *B. burgdorferi* cells per ml of whole blood in humans is approximately 0.1 spirochetes per ml and therefore re-isolation of spirochetes from blood has

demonstrated limited efficacy when using small volumes of blood [145]. Hence, an alternative approach has been proposed to sample blood cultures and test by qPCR for increasing amounts of spirochete DNA [146]. While an enrichment step is practical, the use of qPCR has the potential to introduce false positive results from contaminating *B. burgdorferi* template DNA in the laboratory and typically requires additional protocol steps for nucleic acid purification. iPCR, which herein has demonstrated successful detection of spirochetes directly from whole blood and is much less prone to the same contamination issues as the PCR template is unrelated to *B. burgdorferi* and human DNA, could effectively be used to make a more rapid diagnosis from *B. burgdorferi* infected blood cultures. Future work will focus on improving the limit of iPCR direct detection of spirochetes in blood to achieve a detection sensitivity of 1-10 organisms, as has been demonstrated for other microbial pathogens [122, 147-149]. Furthermore, as *B. burgdorferi* is transiently present in the blood of infected patients the iPCR method may also be adapted for direct detection of spirochetes in synovial fluid and/or cerebral spinal fluid. Direct detection of spirochetes in patient samples is not anticipated to serve as the sole method for diagnosis of Lyme disease, rather in conjunction with sensitive and specific detection of *B. burgdorferi* antibodies.

Contributions of an iPCR-Based Approach Using Recombinant Antigens to Future Automated Lyme Disease Diagnostics

The field of Lyme disease diagnostics is challenged by two main issues, a lack of consistent reagents and the need for a more simplified

objective form of testing [111]. There are currently multiple commercial assays that use a range of antigen types from single recombinant antigens to multiple antigens to whole sonicated organisms. One principal focus for the field has been on the use of purified, recombinant, or synthetic peptides as the source of antigens in immunoassays [111]. Unfortunately, no single antigen has demonstrated sufficient sensitivity and specificity to warrant replacing two-tier testing [111]. Protein expression differences among species and temporal appearance of relevant antibodies to different antigens at various stages of Lyme disease make the choice of a single antigen a difficult task and makes the combined use of antigens an attractive alternative [111]. The results presented here suggest that iPCR combined with the use of recombinant *B. burgdorferi in vivo*-expressed antigens has the potential to provide improved sensitivity of detection in an objective format that can be used to detect multiple host response antibodies and isotypes. Moreover, future translation of this method to an automated point-of-care platform will allow for objective routine testing of Lyme disease patients.

CHAPTER THREE:
SIMPLE QUANTITATIVE DETECTION OF HUMAN LYME DISEASE INFECTION USING IMMUNO-PCR DETECTION OF HOST GENERATED IgG ANTIBODIES AGAINST A SINGLE HYBRID RECOMBINANT ANTIGEN

Introduction

Lyme disease is the most commonly reported tick-borne illness in the United States with approximately 30,000 cases reported to the Centers for Disease Control and Prevention (CDC) each year [12]. New preliminary estimates released by the CDC indicate that the number of Americans diagnosed with Lyme disease each year is closer to 300,000, which are roughly 10 times higher than the annual number reported [13]. This new estimate supports studies published in the 1990s indicating that the true number of cases is between 3- and 12-fold higher than the number of reported cases [150, 151] making Lyme disease a significant health concern within the United States. Accurate diagnosis provides a significant obstacle for the clinical management of the disease and is necessary to differentiate Lyme disease from other diseases with similar clinical presentation. Misdiagnosis of Lyme disease is common due to difficulties in detection of *Borrelia burgdorferi*, the causative agent of Lyme disease [152]. Although a wide range of laboratory diagnostic approaches have been explored, the current accepted method utilizes detection of serological response to *B. burgdorferi* antigens [114].

The current method for detection of Lyme disease in a clinical setting approved by the CDC entails a two-tiered approach using a first-tier enzyme

immunoassay (ELISA) followed by a second-tier immunoblot assay for both IgM and IgG *B. burgdorferi*-specific antibodies using whole cell *B. burgdorferi* lysates, recombinant antigens, or various combinations depending on the commercial kit used [111]. The ELISA provides a quantitative and sensitive first-tier screen but lacks the specificity and broad strain applicability [153] required for a standalone test. The second-tier immunoblot provides a higher level of specificity but currently requires somewhat subjective analysis due to its qualitative nature and lack of automation [154]. A tiered approach has to date provided the most effective means of diagnosing Lyme disease in a clinical setting [111].

Other approaches to diagnosing Lyme disease have been developed including live culture, PCR and additional molecular based methods with no technique surpassing the effectiveness of a serology based approach [111]. In our previous study we demonstrated the use of immuno-PCR (iPCR) for detection of host generated antibodies in a murine model as well as preliminary data using serum collected from Lyme disease patients and healthy controls [155]. Our results indicated that iPCR using *B. burgdorferi* whole cell sonicates and a limited number of *B. burgdorferi* recombinant antigens provided higher sensitivity of detection of *B. burgdorferi* antibodies in infected mice and equivalent sensitivity of detection of *B. burgdorferi* antibodies in Lyme patient serum compared to both ELISA and immunoblot [155].

It is well established that multiple antigens are required for accurate overall diagnosis of the multiple stages and types of Lyme disease [111].

Furthermore, it is critical that the antigens used for diagnosis are demonstrated to have low cross-reactivity for diseases other than Lyme disease. The goals of this study were to 1) determine the range of the levels of background detection of the Lyme disease iPCR assay across a healthy human population, 2) explore a larger subset of antigens for increased sensitivity and specificity and 3) compare the performance of the optimized Lyme disease iPCR protocol with the current 2-tier method of Lyme disease diagnosis.

Materials and Methods

Healthy Human Sera

The current study was approved by University of Central Florida's Institutional Review Board (UCF IRB; FWA00000351, IRB00001138). All procedures and investigators involved in the sample collection process were UCF IRB-approved with Collaborative Institutional Training Initiative training. All donors provided written consent to participate in the current study. Sample collection was undertaken at the UCF campus. UCF is a diverse community of nearly 60,000 students and approximately 8,000 faculty and staff members of various ages, ethnic and racial backgrounds. Individuals were classified for inclusion in the study if they had not been previously diagnosed with Lyme disease, received a Lyme vaccine or lived within the past 10 years in a state with a high incidence of Lyme disease (Connecticut, Delaware, Maine, Maryland, Massachusetts, Minnesota, New Hampshire, New Jersey, New York, Pennsylvania, Vermont, Virginia and Wisconsin). Approximately 10

millilitres of blood were sampled according to the IRB approved protocol from 36 individuals into serum separator tubes, inverted five times to mix the clot activator with the blood and allowed to clot for at least 30 minutes. Serum fractions were collected by centrifugation at 1200 x g for 10 minutes. Serum was further clarified by centrifugation at 9100g for 5 minutes to remove any insoluble material and stored at 4°C for short term or -80°C for long term storage.

Lyme Disease Human Sera Panel

Retrospective, human Lyme disease and healthy control serum samples were kindly supplied by Dr. Martin Schriefer (Centers for Disease Control and Prevention, Fort Collins, CO). The CDC Research Panel I consisted of patient sera collected from 32 individuals including patients with stage 1, 2 or 3 Lyme disease (*n*=12), look-alike diseases including fibromyalgia, rheumatoid arthritis, multiple sclerosis, mononucleosis, syphilis and severe periodontitis (*n*=12) and healthy individuals from both Lyme disease endemic (*n*=4) and non-endemic (*n*=4) areas. All Lyme disease samples were confirmed *B. burgdorferi* culture and PCR positive. The blinded CDC Research Panel II consisted of patient sera collected from 92 individuals including patients with stage 1, 2,or 3 Lyme disease (*n*=32), look-alike diseases including fibromyalgia, rheumatoid arthritis, multiple sclerosis, mononucleosis, syphilis and severe periodontitis (*n*=36) and healthy individuals from both Lyme disease endemic (*n*=12) and non-endemic (*n*=12) areas. Similar to CDC Research Panel I, all Lyme disease samples in CDC

Research Panel II were confirmed *B. burgdorferi* culture and PCR positive. Prior to analysis all serum samples were clarified by centrifugation at 9,100 x g for 5 minutes to remove any insoluble material and stored at 4°C.

Cloning and Expression of Recombinant Antigens Lacking GST Fusion Tags

rGST-BmpA and rGST-OspC were constructed as previously described [155]. In frame glutathione-S-transferase (GST) fusion proteins for BBK19, OspA, DbpA, RevA, Crasp-2 and BBK50 were generated by PCR amplifying the corresponding coding regions without the signal sequences from *B. burgdorferi* genomic DNA using primer pairs 1147 and 1148 (BBK19), 1151 and 1152 (OspA), 1145 and 1146 (DbpA), 1143 and 1144 (RevA), 1149 and 1150 (Crasp-2) or 1043 and 1044 (BBK50) engineered with BamHI and SalI or XhoI restriction sites (Table 2) and Phusion polymerase (New England Biolabs, Ipswich, MA). PCR products were purified (Qiagen, Valencia, CA), digested with appropriate restriction enzymes (New England Biolabs, Ipswich, MA) and cloned into BamHI and SalI or XhoI-digested pGEX-6P-1 (GE Healthcare, Piscataway, NJ) to generate translational fusions with GST at the N-terminus. Subsequent clones were selected and sequence confirmed by sequence analysis. pGEX-6P-1 plasmids carrying *bmpA*, *ospC*, *bbk19*, *ospA*, *dbpA*, *revA*, *crasp-2* or *bbk50* were transformed into a BL21 strain of *E. coli* (Novagen, Billerica, MA).

Table 2. iPCR DNA oligonucleotide sequences used in this study

Oligo number	Oligo ID	Sequence (5'-3')[a]
T1	Template 1 (IgG coupled)	BIOTIN-agcctcagaccaagccagacaactgcctcgtgacgttgctgcccctaccaacgtacccctacgagtcc
T1F	Template 1 Forward	agcctcagaccaagccagac
T1R	Template 1 Reverse	ggactcgtaggggtacgttgg
T1P	Template 1 Probe	FAM-actgcctcgtgacgttgctgcccct-BHQ1
T2	Template 2 (IgM coupled)	BIOTIN-aggaggagggtcaagtcaccaacgctgctccaggccatcgtgctgatctggaccctggatcgagtga
T2F	Template 2 Forward	aggaggagggtcaagtcacc
T2R	Template 2 Reverse	tcactcgatccagggtccag
T2P	Template 2 Probe	MAX-acgctgctccaggccatcgtgctga-BHQ1
1147	BBK19 F	CGGGATCCttttcaaaagattctcgatcacg
1148	BBK19 R	ACGCCTCGAGtcaattgttaggtttttctttcc
1151	OspA F	CGGGATCCaagcaaaatgttagcagcc
1152	OspA R	ACGCCTCGAGttattttaaagcgtttttaatttcatcaag
1145	DbpA F	CGGGATCCggactaacaggagcaacaa
1146	DbpA R	ACGCCTCGAGttagttatttttgcatttttcatcag
1143	RevA F	CGGGATCCaaagcatatgtagaagaaaagaaag
1144	RevA R	ACGCCTCGAGttaattagtgccctcttcg
1149	Crasp2 F	CGGGATCCgatgttagtagattaaatcagagaaatatt
1150	Crasp2 R	ACGCCTCGAGctataataaagtttgcttaatagctttataag
1043	BBK50 F	CGGGATCCatgtgtaaattatatgaaaagcttacaaataaatcgc
1044	BBK50R	CCGCTCGAGttatctagagtccatatcttgcaattt
1084	DbpA_PEPC10 R	AGGTTTTTTTGGACTTTCTGCCACAACAGGgttatttttgcatttttcatcagtaaaagt
1085	C6_PEPC10 F	CCTGTTGTGGCAGAAAGTCCAAAAAAACCTatgaagaaggatgatcagattgc
1023	C6 Bb R	ACGCGTCGACttacttcacagcaaactttccatc

[a] Uppercase letters indicate non-template sequence used for addition of terminal restriction sites, epitope tags or synthetic assembly.

Protein expression was induced by growth of BL21 cells containing the expression construct for each *B. burgdorferi* antigen in 50-100 ml Magic Media *E. coli* expression medium according to manufacturer's protocol (Invitrogen, Carlsbad, CA) for 24 hours at 37°C with aeration. Recombinant protein purification was performed according to the procedures outlined in the Bulk GST Purification Module (GE Health Sciences, Piscataway, NJ). Purified proteins were dialyzed in Tris buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) overnight at 4°C using D-tube dialyzers (EMD Millipore Chemicals, Philadelphia, PA) and two buffer exchanges to remove excess glutathione. Dialyzed proteins were subjected to protease cleavage of the GST tag overnight at 4 °C according to procedures outlined in the PreScission Protease kit (GE Healthcare, Piscataway, NJ). Cleaved proteins were purified from GST and excess protease using two rounds of Bulk GST purification (GE Health Sciences, Piscataway, NJ) and collection of the eluent. Purified proteins lacking a GST tag were concentrated using Amicon Ultra-2 Centrifugal Filter Devices (EMD Millipore Chemicals, Philadelphia, PA) to a volume of approximately 80 µl and stored at 4°C. Total protein was quantified by absorbance spectrophotometry at a wavelength of 280 nm. Recombinant protein purity and seroreactivity was determined by coomassie gel and immunoblot using infected mouse serum. Briefly, 100 nanograms of each recombinant protein were separated by 12.5% polyacrylamide gel electrophoresis. For coomassie staining, gels were incubated in Imperial Protein Stain (Thermo Scientific, Rockford, IL) for 1 hour and destained in deionized water for 1 hour prior to imaging. For immunoblot analysis proteins

were transferred to a nitrocellulose membrane and the membrane was blocked in 5% skim milk and incubated for 1 hour with mouse sera collected 3 weeks post inoculation with wild type *B. burgdorferi* as previously described [155], diluted 1:200 in Tris buffered saline/0.05% tween pH 7.6 (TBST), washed twice with TBST, incubated with HRP-conjugated goat anti-mouse IgG/IgM (Chemicon International, Billerica, MA) for 1 hour, washed twice with TBST and the signal was detected using the SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Scientific, Rockford, IL).

Cloning and Expression of Recombinant DOC Antigen Lacking GST Fusion Tag

In frame glutathione-S-transferase (GST) fusion protein for the DOC hybrid protein was generated using two distinct PCR amplification steps. First, the corresponding coding regions for DbpA and the C6 peptide of VlsE were amplified separately from *B. burgdorferi* genomic DNA with non-template addition of the PEPC10 sequence to each amplicon using primer pairs 1145 and 1084 (DbpA-PEPC10) and 1085 and 1023 (C6-PEPC10), respectively, engineered with BamHI/SalI restriction sites (Table 2). Both PCR products were diluted 100-fold, combined and synthetically assembled into the DOC construct by overlapping PCR using primer pairs 1145 and 1023. Final constructs were sequenced verified and recombinant protein generated and purified as described above for the other *B. burgdorferi* antigens.

iPCR Reagents, Assay and Signal Amplification

iPCR reagents were prepared and assay conducted as previously described [155] with minor modifications. Briefly, iPCR assays were assembled in a two-sided (sandwich) manner as detailed in Figure 10A with the capability to simultaneously capture and report both IgM and IgG host generated antibodies (Figure 10B). Recombinant antigens lacking fusion tags were used to coat magnetic beads for host antibody capture using 10-20 µg of antigen per mg of beads. Beads were resuspended in 500 µl TBST for secondary antibody incubation. Signal amplification by real-time quantitative PCR was accomplished as previously described [155] with the quantification cycle (Cq) for each reaction determined using manual baseline determination (Cycle 10-20) and a manual threshold setting of 1.0.

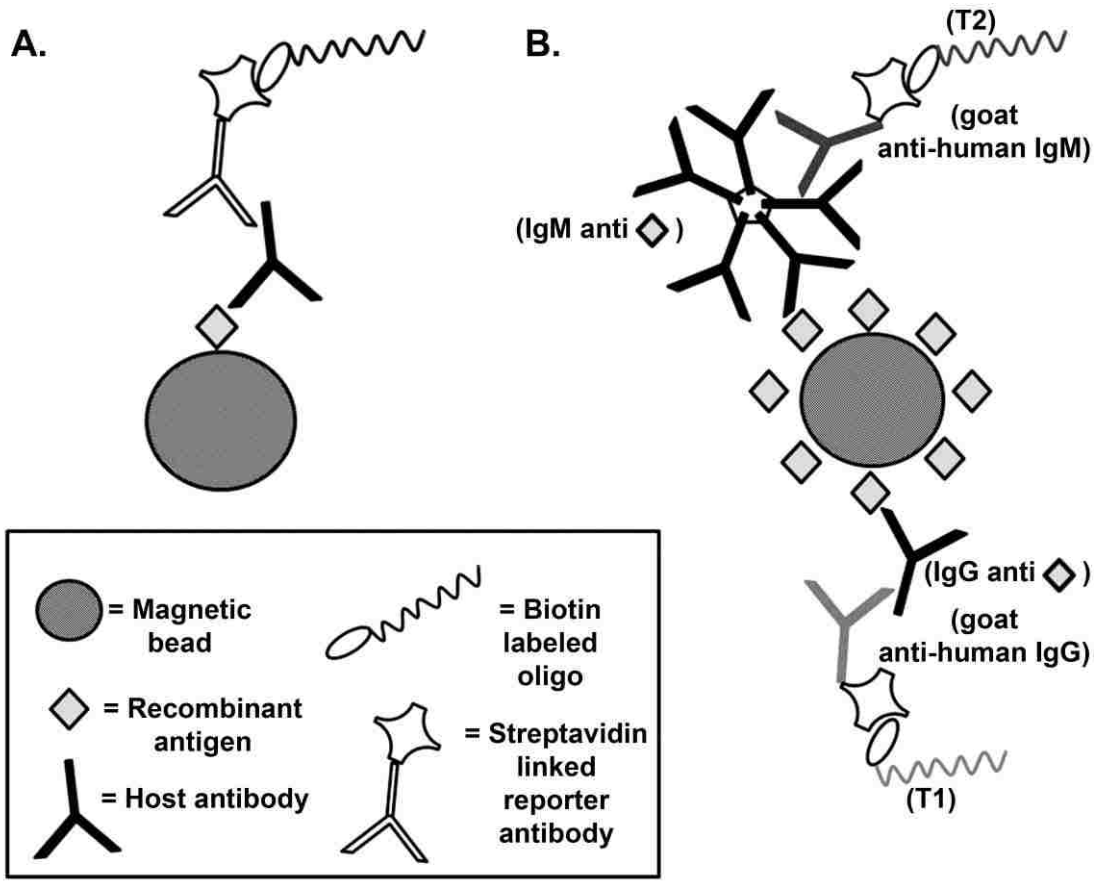


Figure 10. Schematic representation of multiplex iPCR assay for detection of Lyme disease host antibodies using recombinant antigens. A recombinant protein antigen coupled to magnetic beads was used to capture B. *burgdorferi*-specific host-generated antibodies (A). A biotinylated DNA oligonucleotide reporter molecule coupled to a streptavidin-conjugated reporter antibody was amplified by qPCR for detection and quantification. (B) The same antigen coupled beads were used to simultaneously capture both IgM and IgG host generated antibodies which were detected in a multiplex fashion using isotype-specific secondary antibodies coupled to unique reporter oligonucleotides (T1 and T2) similarly amplified by qPCR for quantification.

Positive Threshold Value and Statistical Analysis

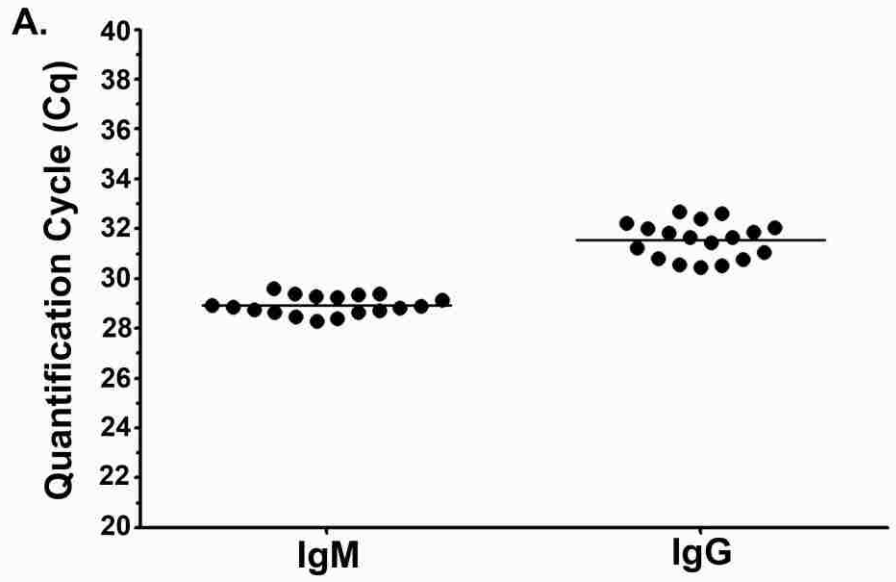
Positive threshold values were established for each individual antigen using an antigen specific multiplier of the standard deviation (SD) above the mean value for a group of sixteen healthy individuals. The antigen specific multiplier was determined using CDC Research Panel I samples as the training set. The antigen specific multiplier was set at a minimal value where the samples from all culture positive individuals resulted in a Lyme disease iPCR positive ΔCq above background. The coefficient of variation (CV) was calculated as the ratio of the SD to the mean. Sensitivity was calculated as the ratio of the number of true positives (correctly identified) to combined true positives and false negatives (incorrectly rejected). Specificity was calculated as the ratio of the number of true negatives (correctly rejected) to combined true negatives and false positives (incorrectly identified). Comparisons were made using the Fisher exact test. P values were 2-tailed and a value of <0.05 was considered significant. All analyses were conducted using Prism GraphPad (GraphPad Software, La Jolla, CA).

Results

IPCR Demonstrates Strong within Assay Precision and Reproducible Background across a Sample Population of Healthy Individuals

We previously demonstrated proof of principle for iPCR detection of human host generated *B. burgdorferi* antibodies using VlsE C6 peptide coated magnetic beads and a panel of serum samples (*n*=36) from Lyme disease

positive and Lyme disease negative patients and healthy controls [155]. This feasibility study was accomplished using a small number of healthy samples (*n*=5) to establish test efficiency and background threshold levels. In an effort to establish a better understanding of the Lyme disease iPCR assay performance, including repeatability and the variability of the background of the assay across a healthy population, the number of replicates and overall sample size of healthy individuals was expanded. Prospective blood samples were collected from consenting individuals without a history of Lyme disease under the approval of the UCF Institutional Review Board. To assess assay repeatability, the serum from a single healthy individual was tested eighteen times using the same reagent preparation lots including DbpA antigen coated beads and oligo-labeled secondary antibodies. The results of this analysis demonstrated low within assay variability for both the IgM- and IgG-specific detection reagents as indicated by standard deviation values for each data set of 0.39 and 0.73, respectively and a coefficient of variation values for each data set of 1.34% and 2.30%, respectively (Figure 11).



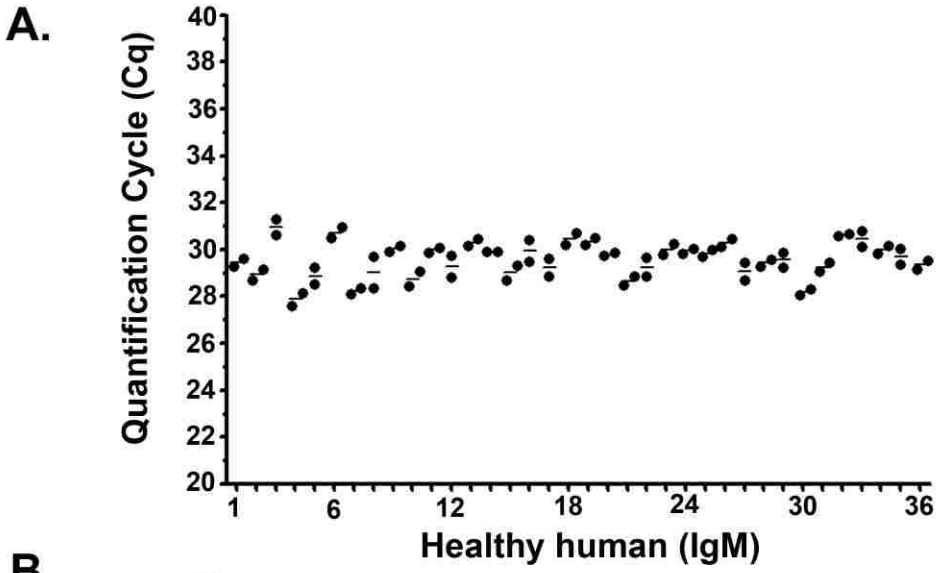
B.

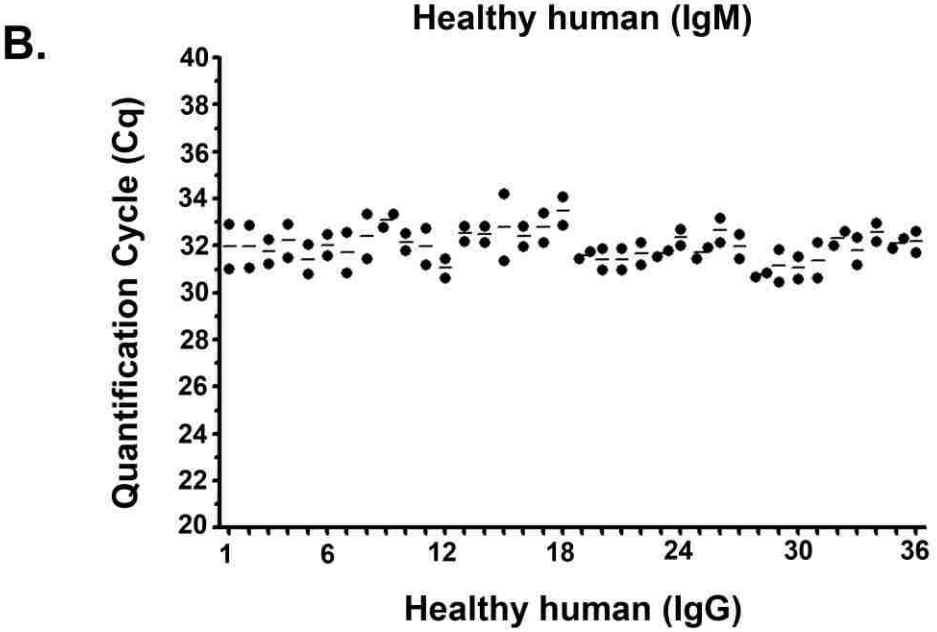
	IgM	IgG
Mean	28.93	31.54
Standard deviation	0.39	0.73
Range	28.30 - 29.61	30.46 - 32.67
Coefficient of variation	1.34%	2.30%

Figure 11. Immuno-PCR magnetic bead protocol demonstrates strong within assay precision. (A) Serum collected from a single healthy individual was assayed 18 times by IgM/IgG multiplex iPCR using recombinant DbpA antigen coupled to magnetic beads. (B) The mean, standard deviation (SD), range and coefficient of variation (CV) (calculated as the ratio of SD to Mean) were calculated for both IgM and IgG. Each dot represents a single replicate and the horizontal line represents the mean Cq value for all replicates for each isotype. The y-axis represents the quantification cycle (Cq) determined by real time quantitative PCR.

To determine the background variability of the Lyme disease iPCR assay across a healthy human population, the sera from 36 healthy individuals were tested in duplicate using magnetic beads coated with the DbpA antigen and the oligo-labeled IgM and IgG secondary antibodies used for the repeatability analysis. Similar to the within sample repeatability analysis, the results of the between sample variability analysis demonstrated

a standard deviation across the population of 0.79 for the background detection of IgM antibodies and 0.84 for the background detection of IgG antibodies and coefficients of variation of 2.66% and 2.63%, respectively (Figure 12).





C.

	IgM	IgG
Mean	29.57	32.00
Standard deviation	0.79	0.84
Range	27.61 - 31.30	30.45 - 34.21
Coefficient of variation	2.66%	2.63%

Figure 12. Lyme disease immuno-PCR demonstrates reproducible background across a healthy human population for both IgM and IgG isotypes using the DbpA antigen. Sera from 36 healthy individuals were assayed in duplicate by multiplex iPCR using both (A) IgM and (B) IgG secondary antibodies and recombinant DbpA antigen coupled magnetic beads. Each dot represents a single replicate per individual with a horizontal line representing the mean value for duplicate serum samples from each individual. (C) The mean, standard deviation, range and coefficient of variation (calculated as the ratio of standard deviation to the mean) is listed for each isotype. The y-axis

represents the quantification cycle (Cq) determined by real time quantitative PCR.

The Mean Background and Standard Deviation Values Across a Population of Healthy Individuals are Unique for Each Lyme Disease iPCR Assay Antigen/Isotype Combination

The analysis of the Lyme disease iPCR assay repeatability and population variability using DbpA coupled magnetic beads demonstrated that the mean background value for the detection of IgM versus IgG antibodies differed by as much as ~2.5 Cq (Figure 11 and 12). Based on this observation, we predicted that depending on the different antigen used each Lyme disease iPCR assay would each result in a distinct mean background Cq value. If true, this finding would impact the determination of the background threshold setting for the assay making it necessary to assign a distinct background threshold for each antigen/isotype combination. To test this hypothesis, a panel of 8 recombinant *B. burgdorferi* antigens either known or suspected to be seroreactive in humans [87-90, 92, 93, 96, 97, 102] was generated and purified as in-frame N-terminal fusions to GST. To eliminate any possibility of antibody cross reactivity to the GST tag, this sequence was proteolytically removed. The purity and antigenicity of each recombinant antigen was demonstrated by SDS-PAGE followed by coomassie brilliant blue staining and immunoblot analysis using pooled sera collected from *B. burgdorferi* infected mice (Figure 13).

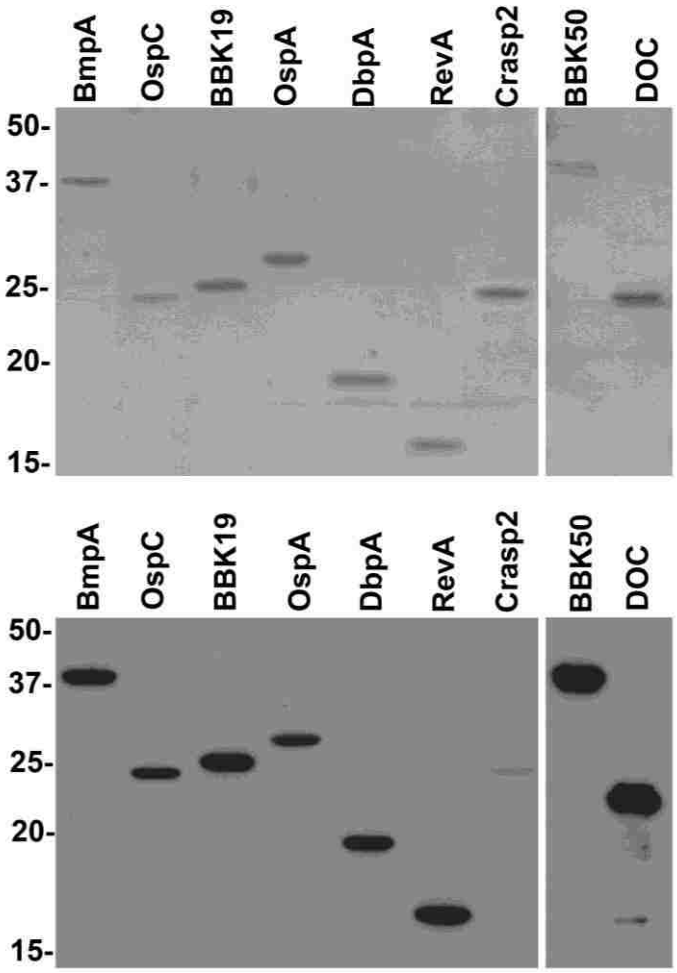


Figure 13. Purified recombinant protein panel exhibits antigenicity in infected mouse serum. Recombinant proteins expressed in *E. coli* were protease treated to remove the GST fusion tag followed by subsequent purification to remove residual GST and protease. Purity and seroreactivity was determined by (A) coomassie gel and (B) immunoblot using infected mouse serum.

Each antigen was coupled to magnetic beads and examined by iPCR for both IgM and IgG background reactivity across sixteen serum samples collected from healthy individuals. As predicted, all antigen/isotype combinations demonstrated unique background values that ranged from a mean Cq of 26.09 to 32.46 for IgM and 25.30 to 36.62 for IgG and a standard deviation of 0.40 to 1.53 for IgM and 0.37 to 1.47 or IgG (Figure 14).

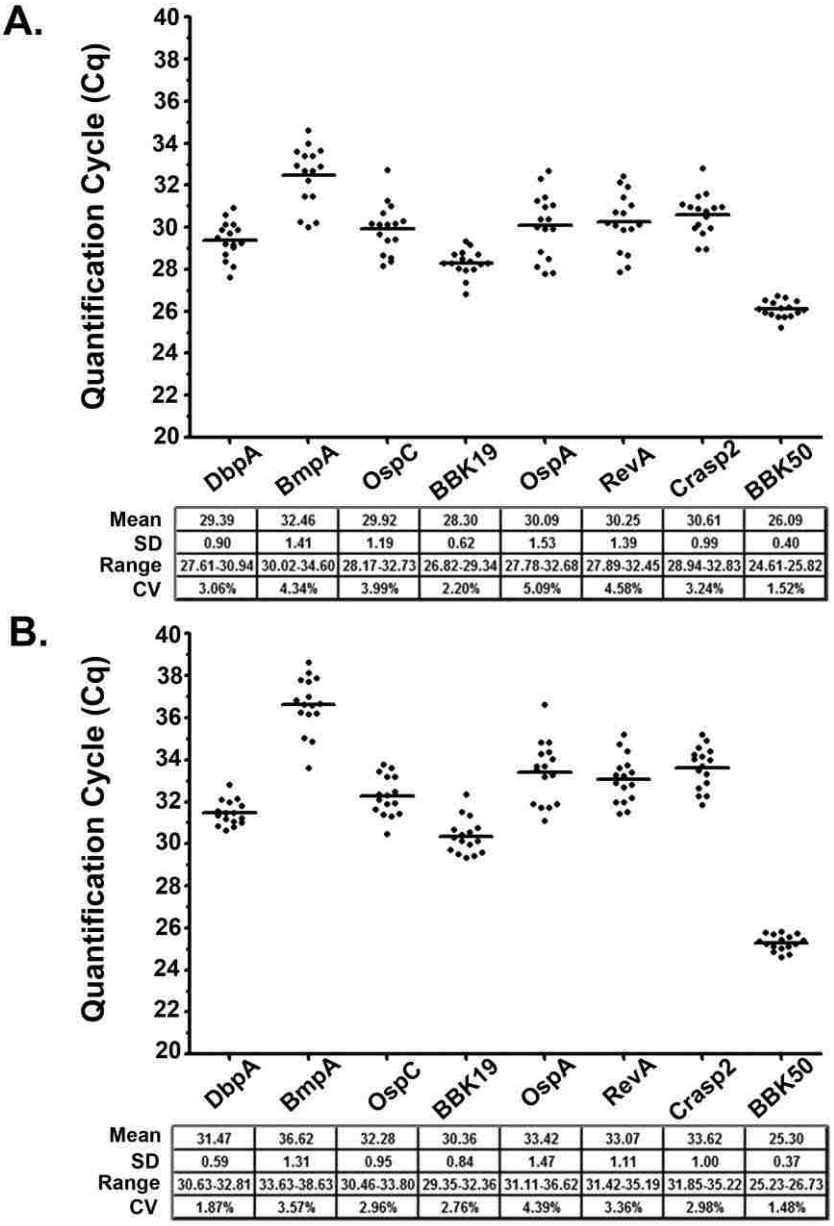


Figure 14. Immuno-PCR demonstrates low intra-antigen background variability for an antigen panel across a healthy human population. Sera from 16 healthy individuals were assayed by multiplex iPCR for both (A) IgM and (B) IgG host generated antibodies against recombinant DbpA, BmpA, OspC, BBK19, OspA, RevA, Crasp2 and BBK50 antigen coupled magnetic beads. Each dot represents a single individual replicate and the horizontal line represents the mean Cq value for all individuals for each antigen/isotype combination. Each antigen mean and standard deviation (SD) are listed. The y-axis represents the quantification cycle (Cq) determined by real-time PCR. The population mean, standard deviation (SD), range and coefficient of variation (CV) is shown for each antigen/isotype combination.

Multiplex iPCR Detection of IgM and/or IgG Host Response Antibodies Against *B. burgdorferi* Using a Panel of Antigens Provides Equivalent Sensitivity and Specificity to 2-tier Testing

Most existing protocols for Lyme disease diagnostics require the use of multiple antigens to diagnose the disease. In an effort to further explore the application of iPCR as a Lyme disease diagnostic, we sought to determine a similar methodology that utilizes a combination of results for different antigens to facilitate diagnosis. The panel of eight *B. burgdorferi* antigens was tested against the CDC Research Panel I collection of sera using multiplex iPCR for simultaneous detection of IgM and IgG host generated antibodies. The same human serum panel had previously been tested according to CDC guidelines by commercial ELISA followed by IgM and IgG immunoblot and classified for 2-tier testing status. Samples were classified as Lyme disease positive by iPCR if they resulted in positive values above the predetermined antigen-specific threshold for IgM or IgG for one or more of the eight antigens tested. Using this criteria iPCR testing provided similar results to 2-tier testing for the Lyme disease patient (Table 3) and non-Lyme disease patient (Table 4) samples with one exception. A single early Lyme disease patient sample, which was deemed 2-tier negative, tested positive by iPCR (Table 3 sample A4). It should also be noted that no single antigen provided iPCR-positive results for all Lyme disease patient samples comprising different stages and types of disease.

Table 3. iPCR using eight antigens demonstrates equivalent results to 2-tier testing for CDC Research Panel I

Disease Stage/Type	Sample	IgM iPCR[a,b]								IgG iPCR[a,b]								iPCR Interpretation	2-Tier Interpretation[c]
		DbpA	BmpA	OspC	BBK19	OspA	RevA	Crasp2	BBK50	DbpA	BmpA	OspC	BBK19	OspA	RevA	Crasp2	BBK50		
Early Lyme-EM (acute)	A1																	Neg	Neg
	A2																	Neg	Neg
	A3																	Neg	Neg
	A4				0.08				0.17									Pos	Neg
Early Lyme-EM (convalescent)	A5			0.21														Pos	Pos
	A6	0.69			0.32													Pos	Pos
	A7			2.21	4.34								5.24					Pos	Pos
	A8			1.60					0.14								0.40	Pos	Pos
Late Lyme (Neurologic)	A9			1.77								3.45						Pos	Pos
	A10				0.09					3.59	2.58		0.14					Pos	Pos
Late Lyme (arthritis)	A11							0.07		4.44	5.49	3.41	0.07	3.74	3.22	3.26		Pos	Pos
	A12					1.13	0.55	1.70		3.87	5.56		1.34		2.98	3.06		Pos	Pos
	Multiplier	2.2	3.2	2.6	3.2	1.3	1.9	4.7	6.6	3.8	4.8	4.8	5.2	2.8	3.7	5.0	3.1		
	Threshold Cq	27.42	27.96	26.82	26.31	28.10	27.62	25.94	23.48	29.24	30.34	27.70	26.01	29.31	28.96	28.60	24.14		

[a] Values shown represent the ΔCq above the positive call threshold Cq value determined using an antigen specific multiplier of the SD above the mean value for a set of healthy individuals for each antigen/isotype combination.

[b] Blank boxes represent iPCR values below the positive call threshold.

[c] 2-tier results established by standard ELISA and IgG/IgM immunoblot

Table 4. iPCR data for CDC Research Panel I for eight antigens and the DOC hybrid antigen in duplicate IgM/IgG

	iPCR IgM[a]										iPCR IgG[a]										2-Tier Interpretation[b]	Tier 1	Tier 2	
	DbpA	BmpA	OspC	BBK19	OspA	RevA	Crasp2	BBK50	DOC2	DOC1	DbpA	BmpA	OspC	BBK19	OspA	RevA	Crasp2	BBK50	DOC2	DOC1		EIA Interpretation	IgM WB Bands	IgG WB Bands
Lyme Stage 1	(1.31)	(1.62)	(3.64)	(0.57)	(1.89)	(2.82)	(1.72)	(1.23)	(1.86)	(1.92)	(1.55)	(0.63)	(3.07)	(0.99)	(2.71)	(2.59)	(3.69)	(1.48)	(1.73)	(1.51)	Neg	Neg	-	30
	(0.69)	(2.03)	(1.17)	(0.68)	(0.19)	(1.21)	(0.33)	(1.15)	(1.08)	(1.21)	(0.86)	(2.78)	(1.57)	(1.73)	(1.00)	(1.76)	(2.60)	(1.39)	(1.40)	(0.92)	Neg	Neg	-	18
	(2.14)	(2.87)	(2.88)	(1.20)	(2.57)	(2.71)	(1.28)	(1.20)	(2.71)	(2.18)	(1.85)	(2.10)	(2.68)	(1.83)	(3.31)	(2.52)	(2.13)	(1.35)	(1.59)	(1.63)	Neg	Neg	-	45,41
	(1.29)	(1.37)	(0.87)	0.06	(0.62)	(0.89)	(0.94)	0.17	(1.84)	(1.90)	(1.77)	(1.51)	(2.13)	(0.83)	(2.20)	(2.04)	(2.47)	(0.76)	(2.17)	(1.93)	Neg	Neg	41,23	66,41
	(1.54)	(3.10)	0.21	(0.84)	(2.30)	(1.94)	(2.18)	(1.24)	(1.68)	(1.43)	(1.46)	(1.43)	(1.16)	(1.66)	(1.78)	(1.61)	(3.37)	(1.50)	0.20	0.73	Pos	Pos	41,23	41,23
	0.69	(0.86)	(0.14)	0.32	(1.33)	(1.38)	(0.31)	(0.63)	(0.48)	(0.10)	(0.27)	(0.07)	(0.29)	(1.21)	(0.74)	(0.25)	(1.57)	(0.90)	1.16	1.32	Pos	Pos	39,23	45,41,23,18
	(1.72)	(2.00)	2.21	4.34	(1.53)	(2.24)	(0.67)	(0.75)	(1.75)	(1.95)	(2.83)	(2.14)	(2.63)	5.24	(2.74)	(2.22)	(3.02)	(1.27)	0.48	0.43	Pos	Pos	41,23	45,41,39,30,23
	(1.46)	(0.92)	1.60	(0.06)	(1.12)	(1.28)	(0.29)	0.14	(1.29)	(1.32)	(2.48)	(1.04)	(1.90)	(1.65)	(3.23)	(2.59)	(1.78)	0.40	0.74	0.28	Pos	Pos	41,39,23	66,41,23
Lyme Stage 2/3	(0.28)	(1.31)	1.77	(0.39)	(0.81)	(1.10)	(2.16)	(0.15)	(0.91)	(0.57)	(1.02)	(0.89)	3.45	(1.43)	(0.63)	(2.16)	(2.09)	(1.33)	1.38	1.52	Pos	Pos	41,39,23	93,41,39,23,18
	(0.38)	(1.82)	(0.25)	0.09	(0.81)	(0.75)	(1.53)	(0.76)	(2.28)	(2.07)	3.59	2.58	(0.87)	0.14	(1.81)	(2.11)	(1.22)	(0.24)	2.57	2.57	Pos	Pos	41,23	93,66,58,45,41,39,30,28,23,18
	(0.19)	(0.38)	(0.08)	(0.13)	(0.03)	(0.22)	0.07	(0.21)	(1.19)	(1.66)	4.44	5.49	3.41	0.07	3.74	3.22	3.26	(0.44)	2.80	2.91	Pos	Pos	41	93,66,58,41,39,23,18
	(0.70)	(0.26)	(1.81)	(0.47)	1.13	0.55	1.70	(0.22)	(0.57)	(0.84)	3.87	5.56	(2.15)	1.34	(2.04)	2.98	3.06	(1.08)	2.74	2.62	Pos	Pos	23	93,66,58,45,41,39,30,28,23,18
Look-alike	(1.59)	(1.36)	(2.36)	(0.68)	(0.71)	(1.03)	(1.02)	(1.66)	(2.17)	(2.00)	(2.70)	(2.41)	(2.59)	(2.42)	(2.69)	(2.50)	(2.32)	(1.56)	(2.14)	(1.76)	Neg	Neg	-	41
	(1.37)	(1.89)	(2.53)	(0.57)	(0.93)	(1.31)	(0.33)	(1.14)	(0.96)	(1.49)	(2.08)	(2.31)	(2.89)	(1.89)	(2.88)	(2.48)	(1.23)	(1.71)	(0.85)	(1.49)	Neg	Neg	-	-
	(1.73)	(2.27)	(2.95)	(0.41)	(3.12)	(2.55)	(2.45)	(2.03)	(2.57)	(2.68)	(2.68)	(1.81)	(2.93)	(1.88)	(4.99)	(3.26)	(3.18)	(1.81)	(2.00)	(2.44)	Neg	Neg	-	66,41
	(1.25)	(3.25)	(0.88)	(1.50)	(1.45)	(0.13)	(1.74)	(1.57)	(2.13)	(2.33)	(2.10)	(3.11)	(1.48)	(2.67)	(3.60)	(1.40)	(2.68)	(1.77)	(2.07)	(2.28)	Neg	Neg	-	-
	(0.07)	(1.50)	(0.29)	(0.04)	(1.21)	(0.88)	(0.36)	(0.03)	(1.34)	(1.47)	(0.17)	(1.83)	(0.16)	(0.77)	(0.14)	(1.21)	(1.28)	(1.23)	(0.90)	(1.33)	Neg	Neg	-	41
	(0.77)	(0.11)	(0.39)	(0.80)	(2.06)	(1.86)	(1.97)	(0.47)	(2.20)	(2.28)	(0.48)	(0.22)	(0.18)	(1.35)	(0.94)	(1.29)	(1.52)	(0.29)	(1.25)	(1.19)	Neg	Neg	-	41
	(1.31)	(1.89)	(2.14)	(0.83)	(2.23)	(0.94)	(1.90)	(0.05)	(0.22)	(0.06)	(1.65)	(1.12)	(1.76)	(1.15)	(2.67)	(3.01)	(3.00)	(0.99)	(1.16)	(1.12)	Neg	Pos	-	-
	(1.72)	(1.31)	(2.08)	(0.15)	(1.53)	(1.38)	(1.67)	(0.89)	(0.95)	(0.93)	(1.53)	(0.52)	(1.28)	(0.78)	(2.18)	(1.06)	(2.31)	(1.16)	(1.06)	(0.94)	Neg	Neg	23	-
	(1.39)	(0.83)	(2.51)	(0.00)	(1.38)	(1.73)	(0.77)	(1.33)	(2.14)	(1.96)	(1.24)	(0.30)	(1.95)	(0.75)	(2.28)	(1.40)	(2.37)	(1.07)	(1.58)	(1.35)	Neg	Neg	-	41
	(0.75)	(0.99)	(0.24)	(0.65)	(0.97)	(0.58)	(0.42)	(1.30)	(1.30)	(1.35)	(1.21)	(0.56)	(0.91)	(1.72)	(0.72)	(0.90)	(1.41)	(1.38)	(1.53)	(1.39)	Neg	Pos	23	-
	(0.50)	(1.87)	(0.42)	(0.41)	(1.28)	(1.04)	(1.39)	(1.24)	(1.88)	(1.68)	(0.42)	(2.02)	(0.31)	(0.96)	(0.91)	(0.89)	(1.67)	(0.81)	(1.11)	(0.66)	Neg	Neg	-	41
	(1.54)	(1.98)	(2.97)	(0.05)	(2.06)	(0.54)	(1.21)	(1.23)	(2.26)	(1.87)	(1.56)	(0.99)	(1.67)	(1.39)	(1.88)	(1.49)	(2.83)	(1.48)	(1.78)	(1.39)	Neg	Neg	-	-
Healthy	(0.73)	(1.64)	(0.06)	(0.44)	(0.74)	(0.71)	(0.35)	(0.39)	(0.45)	(0.36)	(1.20)	(2.12)	(0.61)	(1.68)	(1.27)	(1.50)	(1.22)	(0.91)	(1.13)	(0.59)	Neg	Pos	-	-
	(0.78)	(2.16)	(0.20)	(0.38)	(1.43)	(1.40)	(1.51)	(0.67)	(1.19)	(0.86)	(0.04)	(1.40)	(0.09)	(0.08)	(0.45)	(0.06)	(0.04)	(0.01)	(0.45)	(0.07)	Neg	Neg	-	66
	(0.88)	(1.40)	(1.43)	(0.27)	(0.34)	(0.78)	(0.17)	(0.08)	(1.31)	(1.72)	(1.85)	(1.72)	(1.91)	(1.64)	(2.64)	(2.19)	(2.16)	(1.00)	(1.41)	(1.72)	Neg	Neg	-	-
	(1.23)	(1.67)	(0.61)	(0.10)	(0.91)	(0.74)	(0.37)	(0.62)	(1.22)	(1.44)	(1.82)	(2.59)	(1.51)	(4.14)	(1.80)	(2.72)	(2.25)	(0.96)	(1.40)	(1.38)	Neg	Neg	23	45,41
	(2.10)	(1.83)	(3.27)	(1.16)	(1.38)	(2.66)	(1.35)	(0.96)	(2.60)	(2.38)	(2.83)	(2.38)	(3.08)	(2.15)	(3.07)	(2.59)	(2.79)	(1.51)	(3.79)	(2.26)	Neg	Neg	-	66,41,39
	(0.45)	(1.84)	(0.34)	(0.72)	(0.07)	(0.40)	(0.06)	(1.36)	(1.71)	(1.51)	(1.55)	(2.24)	(1.50)	(1.92)	(1.97)	(2.34)	(2.30)	(0.98)	(2.06)	(1.80)	Neg	Neg	23	45,41
	(1.13)	(2.33)	(0.73)	(0.46)	(0.94)	(0.98)	(0.77)	(1.10)	(1.85)	(1.98)	(1.05)	(1.93)	(1.00)	(1.56)	(1.21)	(1.33)	(1.81)	(1.14)	(1.43)	(1.35)	Neg	Neg	41	41
	(0.96)	(1.79)	(2.19)	(0.56)	(1.14)	(0.89)	(1.26)	(1.00)	(1.66)	(1.98)	(1.36)	(0.06)	(1.38)	(1.53)	(0.74)	(1.20)	(1.69)	(0.95)	(1.64)	(1.20)	Neg	Pos	-	-

[a] Values shown represent the ΔCq above (gray shading) or below (parenthesis) the positive call threshold Cq value determined using an antigen specific multiplier of the SD above the mean value for a set of healthy individuals for each antigen/isotype combination.

[b] 2-tier results established by standard ELISA and IgG/IgM immunoblot

Simplified Single Hybrid Antigen iPCR Detection of Host Generated IgG Antibodies Alone Confirms 2-tier Results for a Panel of Human Serum with Semi-quantitative Determination of Disease Stage

iPCR testing with the panel of eight *B. burgdorferi* antigens showed strong potential as a Lyme disease diagnostic by reproducing the 2-tier test results for CDC Research Panel I samples. Although successful, the use of multiple antigens tested against IgM and IgG increases test complexity by requiring testing of a single sample with multiple antigens. In an effort to further simplify the Lyme disease iPCR approach, we theorized that a single hybrid antigen composed of the immunogenic epitopes of multiple *B. burgdorferi* antigens would provide similar results to testing with a panel of whole individual antigens. To examine the applicability of a single hybrid antigen for iPCR detection of host generated antibodies against *B. burgdorferi* infection, we synthetically constructed a novel hybrid antigen composed of full length DbpA, the PEPC10 peptide (OspC) [156] and the C6 peptide (VlsE) [157] referred to as the 'DOC' antigen (Figure 15A). Similar to the previous eight recombinant antigens, we determined the protein purity and seroreactivity toward *B. burgdorferi* infected mouse sera of the hybrid protein (Figure 13). The range of the background reactivity of the DOC antigen in the iPCR assay was determined using the serum from a group of sixteen healthy individuals (Figure 15B). The results of the between sample variability analysis demonstrated a standard deviation across the population of 0.57 for the background detection of IgM antibodies and 0.51 for the background detection of IgG antibodies and coefficients of variation of 2.31% and 1.94%, respectively. Using iPCR, we then tested the hybrid antigen in duplicate

against the CDC Research Panel I for IgM and IgG reactivity utilizing the results to establish a positive call threshold.

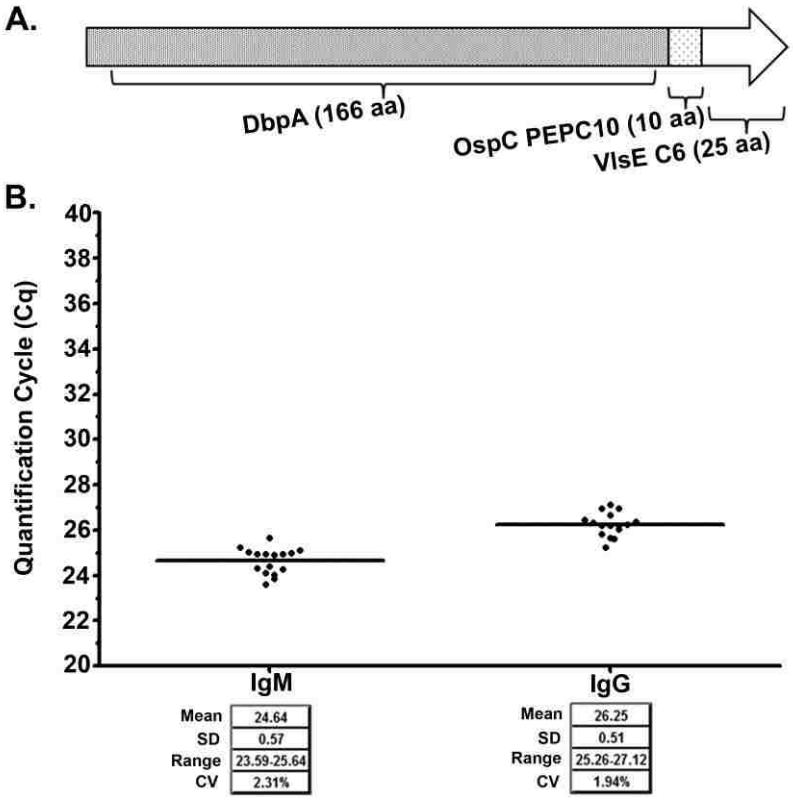


Figure 15. Development of a hybrid antigen for simple detection of Lyme disease. The DOC antigen (A) was assembled using full length DbpA protein fused to the PEPC10 (OspC) and the C6 (VlsE) peptides and (B) was tested by iPCR using DOC coated magnetic beads against sixteen healthy individuals for IgM and IgG for the range of the background reactivity. Each dot represents a single individual replicate and the horizontal line represents the mean Cq value for all individuals for IgM and IgG. The mean and standard deviation (SD) is listed. The y-axis represents the quantification cycle (Cq) determined by real-time quantitative PCR.

The DOC antigen IgG results confirmed all 2-tier positive results (Figure 16B). Interestingly, the iPCR assay using the DOC antigen tested negative for detection of host generated IgM antibodies for all human samples analysed (Figure 16A).

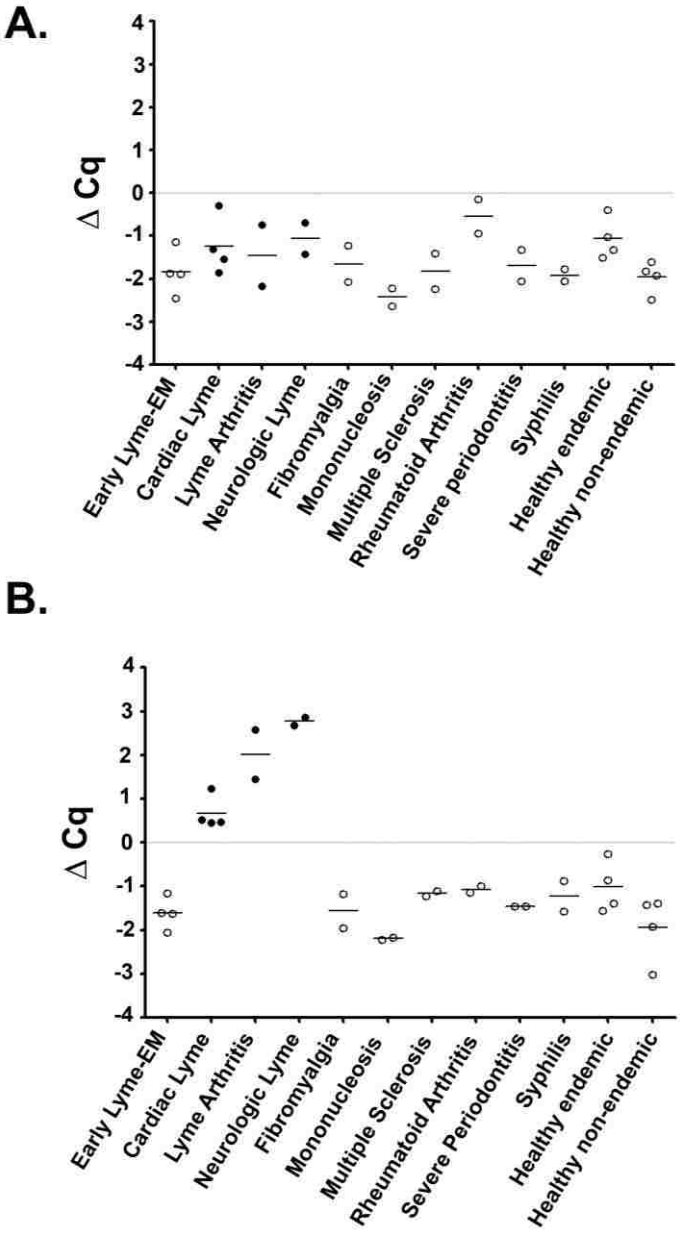


Figure 16. The iPCR assay using the DOC hybrid antigen provides robust detection of Lyme disease. A serum panel composed of 32 samples and consisting of Lyme infected individuals both early (acute and convalescent) and late (neurologic and arthritis) stage as well as look-alike diseases and healthy individuals from endemic and non-endemic areas was tested in duplicate using DOC iPCR for both (A) IgM and (B) IgG reactivity. Each dot represents a single individual replicate and the black horizontal lines represent the mean Cq value for all individuals within each category. Filled circles represent samples that were 2-tier positive with open circles signifying 2-tier negative status. A positive threshold value was established using a multiplier of the standard deviation (SD) above the mean value with the ΔCq threshold (gray horizontal line) representing a value of zero.

Although early and specific detection is the primary goal for any Lyme disease diagnostic, determination of the stage of disease progression would provide additional information to aide in the treatment of the disease. It is logical to assume that the amount of host-generated *B. burgdorferi* antibody will increase with further disease progression. Due to the quantitative nature of iPCR testing, we hypothesized that the amount of anti-DOC host generated IgG antibody would correlate with disease stage. The mean iPCR value was -1.61 ± 0.36 for acute early Lyme disease patients, 0.67 ± 0.38 for convalescent early Lyme disease patients and 2.39 ± 0.64 for late Lyme disease patients for a total of n=4 samples per group. These data suggested a correlation of increasing anti-DOC antibody with disease progression.

DOC Hybrid Antigen IgG iPCR Demonstrates Improved Sensitivity and Higher Specificity Compared to 2-tier Testing for a Blinded Panel of Human Serum Samples

Initial success of DOC IgG iPCR with replicating 2-tier results for a panel of 32 human serum samples provided strong evidence for the application of our approach as a simplified Lyme disease diagnostic. We next sought to perform a larger scale blinded validation analysis of our assay. The CDC Research Panel II composed of 92 samples including sera collected from early, cardiac, arthritic and neurological Lyme disease patients as well as patients with Lyme look-alike diseases and healthy donors was tested by iPCR for host generated IgG antibodies to the DOC hybrid antigen and compared to 2-tier test results (Table 5).

Table 5. iPCR data for CDC Blinded Research Panel II for DOC hybrid antigen IgG

Sample	Sample Group	DOC IgG[a]	iPCR interpretation	2-Tier Interpretation[b]	EIA Interpretation	IgM WB Bands	IgG WB Bands
1	Early Lyme-EM	2.24	Pos	Pos	Pos	41, 39, 23	58, 41, 39, 23, 18
2	Early Lyme-EM	2.20	Pos	Pos	Pos	23	66, 45, 41, 39, 23, 18
3	Early Lyme-EM	2.07	Pos	Pos	Pos	41, 39, 23	41, 23
4	Early Lyme-EM	2.05	Pos	Pos	Pos	41	58, 45, 41, 39, 23, 18
5	Early Lyme-EM	1.59	Pos	Pos	Pos	41, 23	41, 23
6	Early Lyme-EM	1.45	Pos	Pos	Pos	41, 39, 23	66, 45, 41, 39, 23, 18
7	Early Lyme-EM	1.08	Pos	Pos	Pos	41, 39, 23	41, 23
8	Early Lyme-EM	0.80	Pos	Pos	Pos	41, 23	41
9	Early Lyme-EM	0.52	Pos	Neg	Pos	23	66, 41, 23
10	Early Lyme-EM	0.08	Pos	Neg	Equ	-	-
11	Early Lyme-EM	(0.08)	Neg	Neg	Pos	23	66, 41, 23
12	Early Lyme-EM	(0.27)	Neg	Neg	Neg	-	66
13	Early Lyme-EM	(0.58)	Neg	Neg	Pos	23	-
14	Early Lyme-EM	(0.91)	Neg	Neg	Pos	23	41, 23
15	Early Lyme-EM	(1.00)	Neg	Neg	Neg	-	67
16	Early Lyme-EM	(1.01)	Neg	Neg	Neg	39, 23	23
17	Early Lyme-EM	(1.22)	Neg	Neg	Neg	-	23
18	Early Lyme-EM	(1.48)	Neg	Neg	Equ	23	41
19	Early Lyme-EM	(1.50)	Neg	Neg	Neg	23	-
20	Early Lyme-EM	1.14	Pos	Neg	Pos	41	41, 23, 18
21	Neurologic Lyme	2.64	Pos	Pos	Pos	41, 23	45, 41, 23
22	Neurologic Lyme	2.01	Pos	Pos	Pos	41, 39, 23	41, 39, 23
23	Neurologic Lyme	0.00	Pos	Pos	Pos	41, 39, 23	41, 23
24	Neurologic Lyme	(0.26)	Neg	Neg	Neg	41, 23	41, 23
25	Lyme arthritis	3.44	Pos	Pos	Pos	23	93, 66, 58, 45, 41, 39, 30, 28, 23, 18
26	Lyme arthritis	2.96	Pos	Pos	Pos	41	93, 66, 58, 41, 39, 30, 28, 23, 18

Sample	Sample Group	DOC IgG[a]	iPCR interpretation	2-Tier Interpretation[b]	EIA Interpretation	IgM WB Bands	IgG WB Bands
27	Lyme arthritis	2.67	Pos	Pos	Pos	41, 23	93, 66, 58, 45, 41, 39, 30, 28, 23, 18
28	Lyme arthritis	2.62	Pos	Pos	Pos	-	66, 58, 45, 41, 39, 28, 23, 18
29	Lyme arthritis	2.09	Pos	Pos	Pos	23	58, 41, 39, 23, 18
30	Lyme arthritis	1.84	Pos	Pos	Pos	-	93, 66, 58, 41, 39, 30, 23, 18
31	Cardiac Lyme	2.83	Pos	Pos	Pos	41, 39, 23	66, 45, 41, 23, 18
32	Cardiac Lyme	1.37	Pos	Pos	Pos	41, 39, 23	66, 45, 41, 23, 18
33	Fibromyalgia	(0.28)	Neg	Neg	Neg	-	23
34	Fibromyalgia	(0.81)	Neg	Neg	Neg	39	58, 41
35	Fibromyalgia	(1.70)	Neg	Neg	Neg	-	41
36	Fibromyalgia	(1.89)	Neg	Neg	Neg	-	41
37	Fibromyalgia	(1.93)	Neg	Neg	Neg	-	-
38	Fibromyalgia	(2.30)	Neg	Neg	Neg	-	-
39	Rheumatoid arthritis	(0.90)	Neg	Neg	Pos	-	41
40	Rheumatoid arthritis	(1.17)	Neg	Neg	Neg	-	41
41	Rheumatoid arthritis	(1.56)	Neg	Neg	Neg	-	-
42	Rheumatoid arthritis	(1.73)	Neg	Pos	Pos	41, 23	-
43	Rheumatoid arthritis	(1.77)	Neg	Neg	Neg	-	-
44	Rheumatoid arthritis	(2.05)	Neg	Neg	Neg	-	-
45	Multiple sclerosis	(0.55)	Neg	Neg	Neg	39, 23	41
46	Multiple sclerosis	(0.78)	Neg	Neg	Pos	-	41, 23
47	Multiple sclerosis	(1.09)	Neg	Neg	Neg	-	-
48	Multiple sclerosis	(1.11)	Neg	Neg	Neg	39	-
49	Multiple sclerosis	(1.75)	Neg	Neg	Neg	-	-
50	Multiple sclerosis	(2.05)	Neg	Neg	Neg	-	66
51	Mononucleosis	(0.09)	Neg	Neg	Neg	-	39
52	Mononucleosis	(0.28)	Neg	Neg	Pos	-	41, 39
53	Mononucleosis	(0.58)	Neg	Neg	Pos	-	-
54	Mononucleosis	(0.77)	Neg	Neg	Equ	-	41

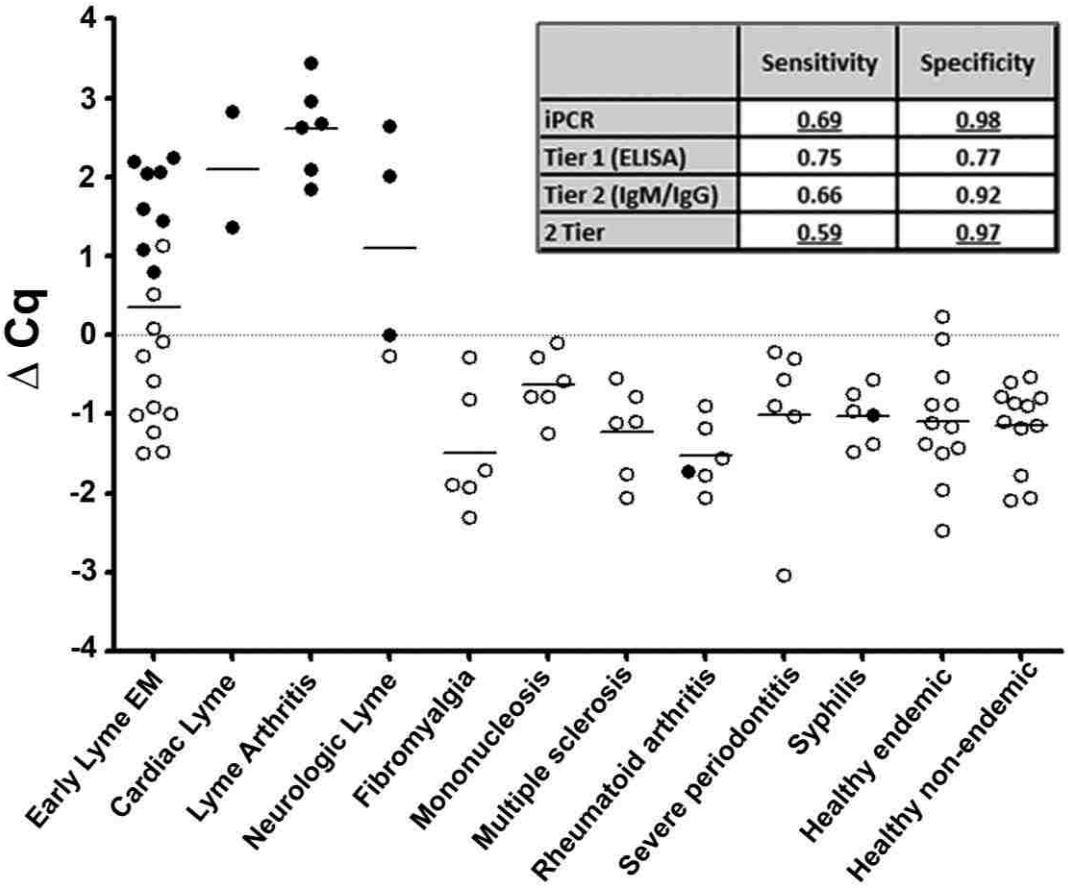
Sample	Sample Group	DOC IgG[a]	iPCR interpretation	2-Tier Interpretation[b]	EIA Interpretation	IgM WB Bands	IgG WB Bands
55	Mononucleosis	(0.78)	Neg	Neg	Neg	-	-
56	Mononucleosis	(1.25)	Neg	Neg	Neg	41, 23	66, 58, 41
57	Syphilis	(0.56)	Neg	Neg	Pos	-	-
58	Syphilis	(0.75)	Neg	Neg	Pos	-	41
59	Syphilis	(0.96)	Neg	Neg	Pos	-	41
60	Syphilis	(1.01)	Neg	Pos	Pos	39, 23	-
61	Syphilis	(1.38)	Neg	Neg	Pos	-	41
62	Syphilis	(1.47)	Neg	Neg	Neg	-	-
63	Severe periodontitis	(0.22)	Neg	Neg	Neg	-	-
64	Severe periodontitis	(0.29)	Neg	Neg	Neg	-	-
65	Severe periodontitis	(0.56)	Neg	Neg	Neg	-	-
66	Severe periodontitis	(0.90)	Neg	Neg	Neg	-	45, 41
67	Severe periodontitis	(1.03)	Neg	Neg	Neg	-	66
68	Severe periodontitis	(3.04)	Neg	Neg	Neg	-	-
69	Healthy endemic	0.23	Pos	Neg	Neg	-	23
70	Healthy endemic	(0.04)	Neg	Neg	Pos	41	66
71	Healthy endemic	(0.53)	Neg	Neg	Pos	-	41, 23
72	Healthy endemic	(0.87)	Neg	Neg	Neg	23	41
73	Healthy endemic	(0.87)	Neg	Neg	Equ	23	-
74	Healthy endemic	(1.11)	Neg	Neg	Neg	-	45, 41
75	Healthy endemic	(1.16)	Neg	Neg	Neg	-	-
76	Healthy endemic	(1.37)	Neg	Neg	Neg	-	-
77	Healthy endemic	(1.42)	Neg	Neg	Neg	-	-
78	Healthy endemic	(1.49)	Neg	Neg	Neg	-	66, 41
79	Healthy endemic	(1.95)	Neg	Neg	Neg	23	-
80	Healthy endemic	(2.47)	Neg	Neg	Pos	23	58, 41, 39, 18
81	Healthy non-endemic	(0.53)	Neg	Neg	Neg	-	41
82	Healthy non-endemic	(0.60)	Neg	Neg	Neg	41, 23	41

Sample	Sample Group	DOC IgG[a]	iPCR interpretation	2-Tier Interpretation[b]	EIA Interpretation	IgM WB Bands	IgG WB Bands
83	Healthy non-endemic	(0.78)	Neg	Neg	Equ	-	-
84	Healthy non-endemic	(0.80)	Neg	Neg	Pos	-	-
85	Healthy non-endemic	(0.86)	Neg	Neg	Neg	-	-
86	Healthy non-endemic	(0.90)	Neg	Neg	Neg	-	58, 45
87	Healthy non-endemic	(1.09)	Neg	Neg	Neg	-	66, 58, 45, 41
88	Healthy non-endemic	(1.15)	Neg	Neg	Neg	-	41
89	Healthy non-endemic	(1.17)	Neg	Neg	Neg	-	41
90	Healthy non-endemic	(1.77)	Neg	Neg	Neg	23	-
91	Healthy non-endemic	(2.06)	Neg	Neg	Neg	23	-
92	Healthy non-endemic	(2.09)	Neg	Neg	Neg	-	-

[a] Values shown represent the ΔCq above (gray shading) or below (parenthesis) the positive call threshold Cq value determined using an antigen specific multiplier of the SD above the mean value for a set of healthy individuals for each antigen/isotype combination.

[b] 2-tier results established by standard ELISA and IgG/IgM immunoblot

Overall, iPCR provided increased sensitivity and specificity compared to 2-tier testing results (Figure 17).



	Sensitivity	Specificity
iPCR	0.69	0.98
Tier 1 (ELISA)	0.75	0.77
Tier 2 (IgM/IgG)	0.66	0.92
2 Tier	0.59	0.97

Figure 17. DOC fusion antigen IgG iPCR demonstrated improved sensitivity and specificity compared to 2-tier testing. CDC Research Panel II was tested in a blinded fashion using DOC iPCR for IgG reactivity. Each dot represents a single individual replicate and the black horizontal lines represent the mean Cq value for all individuals within each category. Filled circles represent samples that were 2-tier positive with open circles signifying 2-tier negative status. A positive threshold value was established using a multiplier of the standard deviation (SD) above the mean value with the ΔCq threshold (gray horizontal line) representing a value of zero. Sensitivity and specificity for iPCR, each tier and combined 2-tier are listed.

iPCR replicated all 2-tier positive results. Moreover, iPCR provided detection of an additional three early Lyme disease samples deemed 2-tier negative, leading to an overall sensitivity for iPCR of 0.69 with a 95% confidence interval (95% CI) of 0.50-0.84 compared to 2-tier at 0.59 (95% CI: 0.41-0.76). The difference in sensitivity was primarily for early stage detection with sensitivity for iPCR at 0.55 (95% CI: 0.32-0.77) and 2-tier at 0.40 (95% CI: 0.19-0.64) for this category of samples specifically. iPCR and 2-tier showed equivalent sensitivity for late stage Lyme samples at 0.92 (95% CI: 0.62-1.0). iPCR detected only a single false positive for a healthy endemic sample providing a specificity of 0.98 (95% CI: 0.91-1.0) as compared to 2-tier testing that detected two false positives for look-alike diseases providing a specificity of 0.97 (95% CI:0.88-1.0). For comparison, the sensitivity and specificity for the ELISA first tier portion of the 2-tier test were calculated to be 0.75 (95% CI: 0.57-0.89) and 0.77 (95% CI: 0.64-0.87), respectively. These data suggested that while the DOC IgG iPCR assay may be less sensitive than the ELISA, our assay has improved specificity over the first tier test.

Discussion

There is an urgent need for development of new tools for improved diagnosis of Lyme disease. This study describes a sensitive, specific and quantitative Lyme disease diagnostic using iPCR detection of host IgG antibody binding to a single recombinant hybrid antigen that demonstrates improved results compared to the 2-tier testing protocol.

The iPCR Approach is a Repeatable Method that Shows Limited Background Variability Across a Healthy Population

Repeatability is a key parameter of any newly developed diagnostic test that provides confidence the test will identify individuals as disease positive or negative in a reproducible manner across the inherent variability of a human population. iPCR has been shown to be a reproducible approach for the detection of other targets [158, 159], although this method generates a background signal in the absence of the analyte being detected [160]. The background signal has been attributed to non-specific binding of the oligonucleotide labelled secondary antibody, similar to results observed for other immuno-diagnostics [125]. Although a number of approaches have been proposed to minimize the level of background amplification [121, 161, 162], no approach to date has proven successful at completely eliminating the background signal. For detection of Lyme disease, we propose that the background signal provides an intrinsic advantage over standard PCR based detection due to the buffer zone created between a negative sample and low level contamination that commonly creates problems for PCR based clinical diagnostic tests. A positive iPCR result is required to generate an amplified signal above the background buffer zone. In addition, critical to the success of this approach is a constant background that remains consistent between sample replicates and standardized across a healthy human population.

In an effort to determine the consistency of the background amplification for the technique we tested the serum from a single healthy individual over eighteen replicates using iPCR and found the standard

deviation of the mean Cq values to be 0.39 and 0.73 for IgM and IgG, respectively with corresponding coefficients of variation of 1.34% and 2.30%. The accepted value for PCR sampling error is ~1 Cq [163] and the coefficient of variation for an ELISA based test is considered good at less than 15% [164]. These data indicate that our iPCR protocol can provide highly consistent and repeatable results across multiple replicates of a single sample. We proceeded to test serum collected from 36 healthy individuals in duplicate for IgM and IgG reactivity using the same antigen to determine variability of the background across a healthy population. Not unexpectedly, compared to the within sample repeatability analysis, we observed a slightly higher standard deviation of the mean Cq values of 0.79 and 0.84 for IgM and IgG, respectively and slightly increased corresponding coefficients of variation of 2.66% and 2.63%. These data indicate that the assay maintains strong repeatability even when compounded with normal human population serum variability. Taken together, these results indicate that the background variability for iPCR detection of host generated antibodies within and across a healthy human population is well within acceptable levels for the technique.

Multiple Antigens are Required for Detection of Lyme Disease Across Multiple Stages/types of Disease

Previous studies using recombinant antigens have indicated that no single antigen tested to date has the capability to diagnose Lyme disease across the multiple stages and/or types of disease manifestation [111]. A panel of eight antigens was generated for use in the iPCR assay. These proteins were selected based on previous studies that identified *B. burgdorferi*

immuno-reactive antigens [102, 165-170]. We first examined the level of variability of the background amplification of each antigen across serum samples collected from healthy individuals for both IgM and IgG isotypes. Each antigen resulted in a unique background amplification mean and standard deviation for each antigen/isotype combination. This indicated that each antigen/isotype combination performed uniquely using the current iPCR protocol. These data provided the necessary parameters including the mean background Cq value and the standard deviation of that mean for determination of an individual call threshold for each antigen/isotype combination. The call thresholds were established as the mean background Cq value minus a multiple of the standard deviation. The multiplier of standard deviation was unique for each antigen/isotype combination and established based on the maximum multiplier that resulted in no false positive calls for the CDC Research Panel I, which served as the training set for optimization of our assay. The ΔCq was calculated as the established threshold call Cq minus the Cq value of the sample. A sample with a ΔCq value ≥0 was deemed positive by iPCR. Using the panel of eight antigens, this approach duplicated 2-tier testing results with a single early Lyme disease patient sample (culture positive) testing positive by iPCR that was negative by 2-tier, suggesting an increased level of sensitivity. Samples from individuals in later stages of the disease (neurologic and arthritis) tended to test positive for multiple antigens.

In addition to detecting the presence of host antibodies and subsequent disease diagnosis, it is important to determine the clinical stage

(i.e., neurological, arthritic, cardiac) of a patient to better understand disease progression. Results from human serum panel iPCR testing classified both late Lyme arthritis samples as strongly positive for IgG using RevA and Crasp2 proteins with all other categories of samples testing negative for the same two proteins. This result suggests that these two proteins may specifically illicit an immune response in arthritic Lyme disease as opposed to other types of Lyme disease. Other studies have shown RevA to be expressed early in human infection [170] and it has been evaluated as a potential vaccine target [171] but no studies have yet linked it to a particular disease type such as arthritis. Crasp2 has been shown to illicit a long-term immune response in a mouse model [172] and explored for use in serological assays [173] but similar to RevA, has yet to be correlated with a disease type. RevA and CRASP-2 have been demonstrated to bind fibronectin and factor H, respectively. Interestingly, a theory has been proposed in which persistence of *B. burgdorferi* infection could be due to the organism coating itself in host macromolecules (i.e., fibronectin) resulting in a decreased immunogenicity combined with protection from complement mediated lysis thus leading to secondary and tertiary stages of the disease including late stage arthritis [174]. This hypothesis would support the preliminary result observed for these two antigens by iPCR testing. However, additional samples would be required to further support these observations.

DOC Single Hybrid Antigen iPCR Detection of Host Generated IgG Antibodies Provides a Simple Quantitative Lyme Diagnostic

Limited studies have shown promising results using antigens composed of multiple antigenic portions of various seroreactive proteins to detect *B. burgdorferi* antibodies in human patient sera [169, 175, 176]. Demonstration of iPCR equivalency to 2-tier testing using a panel of antigens led us to surmise that a more simplified version of the protocol using a single hybrid antigen was likely to be successful. Three antigens known to be seroreactive at different stages of the disease (DbpA, OspC and VlsE) were synthetically joined by combining the sero-reactive peptide portions of OspC [177] and VlsE [178] with the full length DbpA protein into a single recombinant hybrid antigen we termed 'DOC'. The mean background was established for sixteen healthy individuals using DOC and showed little variation (standard deviation of 0.57 and 0.51 for anti-*B. burgdorferi* IgM and IgG antibodies) similar to the full length antigens tested. The DOC antigen was then used to test a CDC Research Panel I for anti-*B. burgdorferi* IgM and IgG antibodies for establishing a positive call threshold. DOC iPCR IgG results demonstrated equivalent results to 2-tier testing with all 2-tier positives showing positive by iPCR. The quantitation of the ΔCq for Lyme disease patients showed a trend with increasing average values from early Lyme acute (-1.61) to early Lyme convalescent (0.67) to late stage Lyme (2.39) suggesting a correlation of the amount of detectable *B. burgdorferi* antibody with disease stage. Surprisingly, DOC iPCR IgM was negative for all samples tested including Lyme disease patient samples. These results indicate that

only testing of the IgG fraction using the DOC hybrid antigen is necessary for Lyme disease diagnosis by iPCR and there exists a potential for determination of the stage of disease based on the ΔCq value.

iPCR testing of the anti-*B. burgdorferi* IgG antibody fraction using the DOC hybrid antigen was successful at duplicating the 2-tier testing results for a small panel of samples. We then proceeded to test a larger blinded panel of 92 samples composed of serum from Lyme disease patients (early, cardiac, arthritis and neurologic), look-alike diseases (fibromyalgia, mononucleosis, multiple sclerosis, rheumatoid arthritis, severe periodontitis and syphilis) and healthy (endemic and non-endemic) individuals (CDC Research Panel II). iPCR demonstrated increased sensitivity (0.69) and specificity (0.98) compared to 2-tier testing (0.59 and 0.97), respectively. iPCR provided the highest level of specificity when compared to each individual tier and was only surpassed in sensitivity by tier-1 ELISA testing (0.75) which also resulted in the lowest level of specificity (0.77). A single neurologic Lyme disease patient tested negative by both iPCR and 2-tier testing. This result is most likely due to the fact that the serum sample was taken 7 days post EM, which was likely too early in infection to produce an adequate immune response.

Application of DOC IgG iPCR as a Future Lyme Diagnostic

For clinical testing, larger cohorts are needed to further standardize the assay and establish the exact cut-off needed to classify the borderline-positive samples as healthy or Lyme disease positive. Currently, the DOC hybrid antigen is composed of *B. burgdorferi* B31 sequences. Amino acid

sequences can vary between strains and species of Lyme disease *Borreliae* by as much as 24% for VlsE C6 [157], 10% for OspC PEPC10C [156] and 44% for DbpA [179]. This may be limiting if an individual is infected with other strains or species. It is likely that the incorporation of additional protein/peptide sequences from other species, such as *B. afzelii* or *B. garinii*, or other strains might further increase the sensitivity of the assay, especially when samples from patients with Lyme disease from Europe and other diverse locations are analysed.

In summary, DOC IgG iPCR shows extraordinary potential as a novel diagnostic tool for identifying host generated antibodies against *B. burgdorferi*. It will be of interest to determine whether this test is useful for monitoring antibody titre changes over time in samples from patients after antibiotic therapy for Lyme disease to determine the stage of disease as well as exploration of specialty testing using this approach to determine the type of disease manifestation.

CHAPTER FOUR:
CONCLUSION

Synthesis and Implications

Lyme disease is the most common tick-borne bacterial disease in North America. According to the Centers for Disease Control and Prevention, Lyme disease is the fastest growing tick-born disease in North America, with greater than 30,000 annual confirmed cases reported in United States and an estimated 300,000 infections every year [13]. *Borrelia burgdorferi* is the causative bacterial agent of Lyme disease in the United States and a spirochete that stains gram negative. Using microscopy, it is typically characterized by its corkscrew morphology and periplasmic flagella. *B. burgdorferi* cycles between small rodents and hard ticks including *Ixodes scapularis* in the Northeastern and Midwestern United States and *Ixodes pacificus* in Western states [180]. The organism does not cause disease symptoms in the tick or the mouse, both of which serve as reservoir hosts. Lyme disease outbreaks typically correlate with seasonal changes in tick activity with the height of transmission during late spring. Humans are not a natural host for *B. burgdorferi* but are infected when fed upon by an infected tick, resulting in disease manifestation following transmission of the organism. Lyme disease is an immunopathologic response to *Borrelia burgdorferi* and has three stages of infection. The first stage is an early, localized infection characterized by erythema migrans (EM) or a ‘bull's eye’ rash that appears 3 to 30 days after a tick bite. This rash symptom is seen in about 75% of the

infected population. Other signs of early stage infection include fatigue, chills, fever, and headache. The next stage of infection is an early, disseminated infection, which results from dissemination of spirochetes to distant tissues like joints, heart, bladder, central nervous system and secondary skin sites. The final stage of infection occurs late and is characterized by arthritis, carditis and meningitis [180]. Antibiotic treatment for a duration of two weeks has shown to be successful following proper diagnosis. If gone untreated, Lyme disease is often difficult to treat in the advanced stages [181]. No accepted vaccine is currently available for Lyme disease so improved methods for diagnosis and treatment are necessary and continue to be important areas of research interest.

Accurate diagnosis of Lyme disease poses one of the greatest challenges to the clinical management of the disease. Misdiagnosis is common as the clinical manifestations of the disease are not unique and detection of a *B. burgdorferi* infection is difficult and prone to misinterpretation [111, 129]. There is great need for the development of improved methods for the definitive diagnosis of Lyme disease. iPCR is a powerful and highly versatile approach for the detection of protein antigens and the host response antibodies that are produced against those antigens [126, 162]. This methodology combines the sensitivity of PCR with the specificity and versatility of ELISA-based protocols [118] and is an excellent technical tool for detection of low level proteins including antibodies.

The dissertation presented addresses the hypothesis that the application of iPCR to detection of *B. burgdorferi* infection will result in an

improved diagnostic method for detection of Lyme disease. The studies used to support this theory focused on the development and application of iPCR for detection of host-generated antibodies to *B. burgdorferi* and comparison of the approach to the currently accepted diagnostic methods for Lyme disease using an experimental mouse model of infection as well as human Lyme patient serum samples.

iPCR Demonstrates the Capability for Both Direct and Indirect Detection of Multiple *B. burgdorferi* Targets

The iPCR methodology is similar to that of a two-sided (sandwich) immunoassay in which the target protein is acquired between a capture antibody or antigen and a reporter antibody (Chapter 2, Figure 4). In contrast to an ELISA, which uses an enzyme/substrate detection system, the detection system for iPCR is quantitative PCR amplification of a specific DNA molecule conjugated to the reporter antibody [118, 120, 126, 162]. PCR amplification of the DNA reporter results in exponential amplification of the output signal allowing detection of rare biomarkers in complex biological samples [118, 122, 149, 182-184]. Similar to enzyme immunoassays, the specificity and versatility of iPCR is determined by the specificity of the capture and reporter antibodies for the target antigen [162]. The flexibility of the iPCR approach was demonstrated by capture and direct detection of intact *B. burgdorferi* (Chapter 2, Figure 9) and indirect detection of host generated antibodies in serum of *B. burgdorferi* infected mice (Chapter 2, Figure 6) using a similar magnetic bead capture methodology. Similarly, it was shown that a variety of antigens including intact spirochetes (Chapter 2, Figure 6) as well as single

recombinant antigens (Chapter 2, Figure 7) could be used to capture host-generated antibodies in the serum of *B. burgdorferi* infected mice. The flexibility of the iPCR approach is advantageous and important for a disease such as Lyme disease where measurement of a single disease marker is unlikely to provide a comprehensive diagnostic assay. Although multiple target detection is compatible with other diagnostic systems [185], it has been suggested that with careful selection of multiple capture/reporter antibody combinations along with unique DNA reporter molecules, iPCR assays may be capable of concurrent detection of several protein biomarkers in the same sample [186]. This possibility of multiplex analysis of a single sample is particularly intriguing for the development of a detection method for Lyme disease biomarkers as it would provide the ability to identify and quantitate the presence of several *B. burgdorferi* antibodies and/or antigens at the same time (Chapter 3, Figure 10B) and ultimately could lead to the ability to determine the specific *Borrelia* species that caused the infection and/or to determine the disease stage of the patient. We have demonstrated that using iPCR both IgM and IgG host antibodies generated against a *B. burgdorferi* infection could be captured and detected simultaneously using magnetic beads coated with single *B. burgdorferi* antigen (Chapter 2, Figure 7 and 8). It is intriguing to extend this same concept to detection of not only additional host antibody isotypes such as IgA [185] or IgE [187] but also provide the capability of concurrent direct detection of *B. burgdorferi* antigens within the same sample. This same multiplex-based approach would be difficult if not impossible with other diagnostic methods that typically employ a single

reporter molecule such as an enzyme that is incapable of differentiating multiple targets in a single sample. It is important to note that multiplex detection of multiple antigen types for purposes of increasing specificity, species identification and/or disease staging would require improvements on the current iPCR protocol described here. The current protocol does not differentiate between antigen types conjugated to the magnetic beads but only the host antibody isotypes that bind a single antigen type. Methods for determining antibody binding to multiple bead conjugated antigens would require a more sophisticated approach that would combine the capability to distinguish both bead type and antibody binding status simultaneously. This could theoretically be accomplished by combining a digital PCR approach [188] with established methods for multiplex microsphere analysis [189] in a single platform. Although technically challenging, this could provide a viable method for multiplexing both antigens as well as antibody isotypes for a more complete picture of host immune response.

iPCR Utilizing Intact *B. burgdorferi* Shows Improved Sensitivity Using a Mouse Model

In addition to the ability to detect multiple targets, sensitivity is a key parameter required for diagnosis of Lyme disease particularly in the early stages of disease when levels of host antibody can be quite low. Because of its signal amplification power, iPCR demonstrates a 100-10,000 fold increase in the typical detection limit of the ELISA [120]. The specificity and sensitivity of iPCR makes it a highly effective method for diagnosis of infectious diseases. Indeed, iPCR has been used for ultrasensitive detection of viral

and bacterial pathogens and antibodies [121, 125, 147-149, 190-194]. In recent years vast improvements have been made to iPCR protocols, surmounting many of the difficulties, such as high background and lack of reproducibility, which have impeded the use of iPCR as a microbiological diagnostic tool in clinical laboratories [127]. It was demonstrated that iPCR using intact spirochetes to capture host-generated antibodies provided earlier detection (day 11) than either a commercial ELISA (day 14) or standard immunoblot (day 21) for a murine infection model (Chapter 2, Figure 6). These data have strong implications for iPCR detection of Lyme disease in humans. Typically, detection of host antibody response is less successful in early stage of Lyme disease due to extremely low levels of circulating antibodies to the spirochete. A diagnostic method such as iPCR that demonstrates more sensitive, and hence earlier, detection of *B. burgdorferi* antibodies provides the opportunity to begin treatment in a timelier manner which will ultimately minimize complications due to infection.

iPCR Using Recombinant Antigens Further Improves Sensitivity and Demonstrates Strong Correlation with a Commercial ELISA For Human Serum Samples

The initial iPCR assay design for capturing host antibodies against *B. burgdorferi* employed magnetic beads coated with intact spirochetes. Although this method proved successful, it resulted in only a small positive difference between uninfected and infected mouse serum. These data suggested that only a minor percentage of the proteins exposed on the surface of the *B. burgdorferi* were antigenic and therefore capable of

capturing host antibodies generated in response to a *B. burgdorferi* infection. It therefore seemed reasonable to hypothesize that the use of specific recombinant *in vivo*-expressed *B. burgdorferi* antigens as the bait for the host response antibodies would likely provide improved sensitivity. The basis for this hypothesis was that magnetic beads coated in specific *in vivo*-expressed antigens would provide optimal presentation of an increased concentration of a specific target for host antibody capture as opposed to a reduced concentration of multiple targets that would be presented on the surface of a whole spirochete. This was found to be the case as demonstrated by detection of host response antibodies against *B. burgdorferi* at day 7 post inoculation in the mouse model of infection using magnetic beads coated in two distinct recombinant antigens (Chapter 2, Figure 7) as opposed to at day 11 post inoculation using magnetic beads coated with intact spirochetes (Chapter 2, Figure 6). Although specificity was not analysed in these experiments, it also seemed likely that the use of single *B. burgdorferi*-specific *in vivo*-expressed antigens would reduce the opportunity for cross-reactivity, which may occur with a higher frequency when the antibody capture systems uses antigens that are highly conserved across microorganisms, such as flagellar proteins [195]. The use of carefully selected *B. burgdorferi*-specific antigens provided the opportunity to reduce or potentially eliminate cross-reactivity, screen for the antigens that demonstrated the highest sensitivity and potentially apply a select multi-antigen approach that may detect antibodies developed against antigens differentially expressed across the disease spectrum. As stated earlier, host generated antibodies during a *B.*

burgdorferi infection can vary significantly by the type and stage of infection, strain and species of the infecting spirochete as well as the range of immune responses elicited by different individuals. All in all, our preliminary data using a mouse infection model demonstrated the feasibility and strong performance of iPCR-based detection of *B. burgdorferi* antibodies in infected animals. The next step in development of the iPCR-based assay for detection of Lyme disease was to determine applicability of the approach for testing in human samples.

To determine preliminary feasibility for Lyme disease testing of human samples with our assay, the next step in development was to determine assay performance for a small cohort of individual samples. Although promising, the improved sensitivity the iPCR-based method demonstrated over current methods with a mouse model may not accurately predict the performance and varied background encountered when testing human patient samples. For instance, a population of individuals from different parts of the country would likely be exposed to a number of different strains and present potentially different immune responses to the same strain. In addition, different immune histories (*i.e.*, exposure to other pathogens) could also potentially affect test specificity, as antigens from microorganisms other that *B. burgdorferi* have the possibility of generating antibodies that are cross reactive with *B. burgdorferi* antigens. To explore these issues, preliminary testing utilized a panel of human serum samples from Lyme positive and negative individuals whose disease status was determined by the Centers for Disease Control using a commercial ELISA for combined IgM/IgG reactivity to the VlsE C6 antigen.

iPCR analysis of the human panel for IgM and IgG individual reactivity using the VlsE C6 peptide antigen demonstrated strong agreement with the commercial ELISA (Chapter 2, Figure 8) and as mentioned earlier was able to separately quantitate both IgM and IgG response as opposed to the combined IgM/IgG measurement of the commercial ELISA. All samples positive by ELISA resulted in a positive iPCR call for either IgM or IgG. More importantly, a small subset of samples that tested equivocal or negative by ELISA was found to be positive by iPCR. This result further supported the earlier mouse model observation of increased assay sensitivity for iPCR compared to existing methods. In addition, iPCR demonstrated no false positive results for non-Lyme disease and healthy individuals suggesting high overall specificity for the assay. Due to the small sample size, these results were considered preliminary and required additional testing of human samples to support these conclusions. Nonetheless, the overall results provided strong evidence for iPCR applicability to Lyme disease testing in human samples as a more sensitive method for indirect detection of host generated antibodies.

iPCR Provides a Potential Method for Direct Detection of *B. burgdorferi*

Indirect detection of host immune response by ELISA and immunoblot is the current accepted method for diagnosis of Lyme disease [106]. PCR detection is not recommended under CDC guidelines and culture of the organism from patient blood or tissue is not typically undertaken in a clinical setting [111]. This is primarily due to the fact that *B. burgdorferi* spirochetes

are found transiently in blood, in such low numbers (0.1-1.0 cfu/ml) and require more specialized culture conditions than other organisms [111]. Due to the fact that iPCR has demonstrated success at detection of low levels of organism in bodily fluids for other pathogens [121, 122], it was important to determine the potential for applying the same approach for direct detection of *B. burgdorferi* in blood. Results with a mouse model (Chapter 2, Figure 9) demonstrated that direct capture and detection of *B. burgdorferi* whole organism from blood using iPCR did not reach adequate levels of sensitivity needed based on the predicted low levels of cultivable cells per millilitre of blood in an active human infection. The iPCR method reproducibly detected 1,000 spirochetes/ml; however, detection of spirochetes in human blood would require at least 1000-fold greater sensitivity. There exists potential to use iPCR as opposed to PCR for earlier detection of enriched blood culture positive samples as it not only directly detects *B. burgdorferi* proteins but also provides minimal chance of false positive results due to laboratory contamination, which is a major challenge for PCR detection methods. Additional method development for this purpose would be required and would include testing of alternative antibodies for increased capture sensitivity and protocol optimization for spirochete capture in blood culture medium.

The preliminary work described in the first section of this dissertation provides the initial report for the first successful application of iPCR for indirect detection of Lyme disease. Methodologies and current limitations for detection of both host response antibodies to a *B. burgdorferi* infection and the spirochete itself were demonstrated suggesting potential applications as a

new and more sensitive Lyme disease diagnostic using primarily indirect detection of host generated antibodies. With initial success in human samples, the next stage of development comprised expanded development of the assay and qualification for testing of human Lyme samples.

iPCR Assay Multi-Antigen Development and Qualification as a Human Lyme Diagnostic

With promising results for human sample testing with our iPCR assay, the next step in development was to determine the repeatability and background signal of the iPCR assay for human samples collected from healthy individuals. iPCR, similar to other immuno-based detection methods such as ELISA, results in a normal background signal attributed to non-specific binding of the detection reagents to the solid support matrix. The presence of a background signal makes it important to determine the variability of the background signal for both the technique itself as well as normal variation within the healthy human population. Intra-assay variation was tested by examining multiple replicates of the same serum sample for both IgM and IgG reactivity with a single antigen (Chapter 3, Figure 11), which resulted in strong repeatability for the assay itself. This result was expected based on a small number of replicate samples tested during earlier development work. Of more interest was the variation across a normal human population to determine if the background was indeed reproducible across groups of healthy individuals. A normal background is important for immunoassays like iPCR due to the need to establish the range of values for healthy individuals to determine the threshold cut-off value for the assay that

distinguishes a negative from a positive result. Intra-assay human population variability (Chapter 3, Figure 12) demonstrated a slight increase above the intra-assay technique variability. This result is not unexpected as different individuals with varying immune histories would likely not provide the same background values. Following establishment of iPCR technique repeatability for human samples, the next step was to focus on improving the host antibody capture capabilities of the assay.

Because of the limitations of direct detection of *B. burgdorferi* in patient samples, the majority of current Lyme disease diagnostics rely on detection of host response antibodies to *B. burgdorferi* infection as recommended by the CDC [114]. The first-tier ELISA is the most common type of test performed to detect antibodies against *B. burgdorferi* [111] but this method does pose some challenges to the clinical diagnosis of Lyme disease. The major drawback to the approach is a lack of required standardization which leads to variation within and between commercial kits which can increase the potential for misdiagnosis [111]. Antibody capture using whole-cell sonicates of *B. burgdorferi* as the capture antigen(s) tends to lack specificity due to the presence of conserved, highly cross-reactive antigens [111]. An additional challenge to the accurate detection of Lyme disease is that there are multiple *Borrelia* species that are able to cause the disease [180]. Genetic variability has been documented across isolates [65, 111, 196-198], which suggests that different species and different clinical isolates of the same species may have distinct antigen expression profiles resulting in discrete serological patterns that may not be detectable by single antigen ELISA methods [111]. As a

result, immunodiagnosis of Lyme disease is highly dependent on antigen selection. For this reason, a panel of multiple antigens was examined using iPCR to identify those antigens that demonstrate high sensitivity and specificity in our assay.

A group of antigens previously shown to be sero-reactive in mammals (mouse or human) were expressed as recombinant proteins in *E. coli*. iPCR background signal was then established for each antigen using a sample of healthy individuals. Each antigen/isotype combination provided a unique mean amplification cycle and standard deviation (Chapter 3, Figure 14) for the cohort of healthy samples. For any quantitative assay that generates a background signal in healthy samples, determining the positive call threshold first requires establishment of the mean value and standard deviation for healthy individuals. Typically, three times the standard deviation above the mean is applied for determining the call threshold in ELISA based assays [164]. Although the threshold value cut-off for iPCR was determined using three times the standard deviation of the background amplification of serum from healthy individuals in the initial stages of development of the assay (Chapter 2, Figures 6-8) it became apparent that due to the variation in means and standard deviation values between antigens and antibody isotypes, each antigen/isotype pair would require a unique empirically determined multiplier. It was determined that the multiplier would be established by testing a panel of known Lyme positive and negative samples, supplied by the CDC, for each antigen/isotype combination and adjustment of the standard deviation multiplier to a minimal value that would correctly identify the status of all

positive samples. Hence, the eight recombinant proteins were tested against a training panel of 32 human samples including Lyme patient samples from different stages/types of disease, look-alike diseases and healthy endemic and non-endemic controls (CDC Research Panel I). The disease status for each sample had been established previously by standard two-tier testing using a first tier ELISA and second tier immunoblots for IgM and IgG. iPCR confirmed all two-tier positive samples with an additional early Lyme sample testing positive by iPCR but negative by two-tier analysis (Chapter 3, Table 3 and Table 4). Similar to the higher sensitivity observed for iPCR detection of *B. burgdorferi* antibodies in the previous human panel and mouse model, this result further supported higher sensitivity detection of host generated antibodies compared with two-tier testing. All samples negative by two-tier were similarly confirmed negative by iPCR also demonstrating the strong specificity of the approach.

The current national guidelines for serological diagnosis of Lyme disease recommend two-tier testing, in which a positive ELISA is followed by immunoblot analysis for specific IgM and IgG antibodies [114]. Although the two-tier protocol has improved diagnosis of Lyme disease [111], analysis of immunoblot results requires technical expertise and is prone to subjectivity leading to potential misinterpretation [154]. In addition to increased sensitivity and specificity, a goal for examining the iPCR approach as a method for diagnosing Lyme disease was to apply a technique with objective quantitative results with minimized technical complexity as a potential to replace two-tier testing. Although successful at demonstrating increased sensitivity, an iPCR

protocol that requires the use of a panel of individual *B. burgdorferi* antigens would impart an undesirable complexity to the assay. This is due to the fact that each antigen would require testing of a separate fraction of serum for each individual. Although IgM and IgG could be tested simultaneously due to the multiplex capability of iPCR, the existing panel of eight antigens would require testing of eight aliquots for each individual resulting in a more complex testing and analysis scheme. A more efficient solution would be to combine appropriate antigens into a single hybrid antigen to further minimize the complexity of the iPCR method for Lyme disease.

Numerous ELISA and immunoblot Lyme disease diagnostic methods have been developed using specific *B. burgdorferi* recombinant antigens [111]. Strong assay sensitivities have been shown for other Lyme disease diagnostics when multiple purified antigens are used in combination [144, 169, 199]. Moreover the data presented herein as well as the data from other groups demonstrate that there is no one single *B. burgdorferi* antigen that appears to be diagnostic for Lyme disease (Chapter 3, Table 3 and [111]). As opposed to utilizing a combination of full length proteins, our strategy involved expressing a recombinant hybrid protein using a design scheme that coupled known immunodominant peptides to a highly expressed, small and established seroreactive protein. The DbpA antigen was selected as the full length “anchor” antigen, which has been shown to maintain uniformly high antibody titers in non-human primates throughout the course of disease [107, 200]. The immunodominant peptides for both the VlsE and OspC antigens have been mapped [201, 202], studied [203, 204] and utilized in other

diagnostic formats [205, 206]. The synthetic peptide C6, which represents the invariable region of the VlsE protein is a strong target for IgG antibodies early in Lyme disease progression [144, 178]. The OspC-derived peptide, PEPC10, also has demonstrated a strong immune response in Lyme disease patient sera during early stages of infection [144, 177, 203]. We generated a recombinant hybrid protein that coupled the amino acid sequences of PEPC10 and C6 to the C-terminus of the full length DbpA protein (Chapter 3, Figure 15A). The mean background and standard deviation in a healthy human population was determined for the hybrid antigen we termed 'DOC' (Chapter 3, Figure 15B).

The DOC antigen was then tested in a similar manner as the panel of eight antigens for both IgM and IgG iPCR reactivity against the panel of 32 human sera from CDC Research Panel I. The DOC IgG results using iPCR confirmed two-tier testing results for positive samples with no discrepancies (Chapter 3, Figure 16). Additionally, all look-alike disease and healthy samples tested similarly tested negative by both iPCR and two-tier testing (Chapter 3, Figure 16). Surprisingly, all samples tested negative by DOC IgM iPCR for Lyme disease patient, look-alike disease and healthy samples. Taken together, these results indicated that only testing of the IgG fraction of a sample was required using the DOC antigen and iPCR for 100% correlation with the two-tier results supplied by the CDC. Although surprising, this result indicated a unique and strong benefit to our approach. Depending upon the stage of infection and antigen expression pattern, *B. burgdorferi* may elicit IgM and/or IgG antibody production [207]. The guidelines for immunoblot

interpretation for two-tier testing state that IgM or IgG criteria [99, 110] may be used in the first month of infection. However, immunoblot interpretation is then limited to IgG criteria only after 4 weeks following disease onset, as IgM has been shown to persist post-treatment despite resolution of the infection, making interpretation difficult [98, 207]. This means the time-sensitive use of IgM may not only limit assay sensitivity in the event that IgG antibodies have not fully developed at time points just beyond 4 weeks of infection [208] but also contribute to the complexity, cost and convoluted analysis of the two-tier method. Our preliminary result with the DOC antigen IgG reactivity indicated that only a single tier single isotype test was required to confirm two-tier testing resulting in a significantly simplified protocol for Lyme disease testing that avoids the controversy associated with IgM interpretation.

Beyond IgM interpretation, immunoblot analysis for Lyme disease is subjective and provides only qualitative results for host antibody levels. iPCR, due to the incorporation of quantitative-PCR, provides a means for quantitatively determining the level of host generated antibodies in a serum sample similar to the first-tier ELISA. Beyond confirmation of overall two-tier results, the values established for each positive sample by iPCR for the panel of 32 samples appeared to correlate with disease stage (Chapter 3, Figure 16). For instance, early stage Lyme samples ranged from 0.45 to 1.24 with an average ΔCq of 0.67 (SD=.038). Later stage Lyme samples (neurologic and arthritis) had a ΔCq range of 1.45 to 2.86 with an average of 2.39 (SD=.64). This equates to slightly more than 3-fold higher antibody titres on average in later stage disease samples. These early results suggest the

possibility of applying iPCR for quantitatively estimating the stage of disease progression. This could provide valuable information to help address difficulties in treating Lyme disease at later stages of progression. These results should be considered preliminary and would be further supported with additional testing of well-characterized human samples.

Following successful testing of DOC IgG iPCR using a panel of known human serum samples, we next tested our optimized assay against a larger blinded panel of 92 human serum samples (CDC Research Panel II). The panel was similarly composed of samples from confirmed Lyme disease patients, patients with look-alike diseases and healthy individuals from endemic and non-endemic areas. Confirmation of positive Lyme patient status was established by the presence of single or multiple EM, culture re-isolation of live *B. burgdorferi* organism and *B. burgdorferi* locus specific PCR from EM skin samples. The results from these analyses demonstrated the optimized single antigen approach was capable of detecting all two-tier positive samples with an additional three early Lyme disease patient samples detected by iPCR that were not detected by the two-tier protocol providing a sensitivity of 0.69 compared to 0.59 for two-tier testing (Chapter 3, Figure 17). Only a single false positive was observed for iPCR compared to two false positive samples for two-tier testing providing a slightly higher specificity (0.98 compared to 0.97). These results confirmed the trend evident in both the mouse model as well as earlier testing with other human panels, that iPCR is a more sensitive approach than two-tier testing for diagnosis of Lyme disease in human serum samples. Additionally, iPCR results for specificity were also

slightly improved compared to the existing two-tier protocol. Taken together, these results provide strong support for further exploring the potential to replace current complex and labour intensive two-tier Lyme disease testing with the simple, cost effective, objective and quantitative method of single hybrid antigen IgG iPCR.

Impact on Human Lyme Disease Diagnosis

Certain sectors of society including individuals within the medical community have referred to Lyme disease as a chronic infection that is difficult to treat and in some cases can require prolonged antibiotic treatment for later stage disease. Although the CDC has issued recommended metrics for diagnosing individuals infected with *B. burgdorferi* [106], diagnoses are at the physician's discretion. Improper analyses of diagnostic test results from immunoblot analysis alone or the interpretation of IgM immunoblot banding patterns beyond four weeks of infection have resulted in controversial determinations of disease status. In response to incorrect test analysis, suspected Lyme disease patients may undergo expensive, long-term intravenous antibiotic treatments. This is in direct contradiction to results from more recent clinical trials [209] that found no significant difference in the outcome for Lyme disease positive or negative patients for prolonged antibiotic treatment as compared with placebo. It has also been established that extended courses of antibiotic therapy administered beyond the recommended time course can actually negatively impact a patient's health status [210]. A more objective and less complex diagnostic test for Lyme

disease, such as out iPCR assay, may provide a more concise laboratory result reducing the opportunity for misdiagnosis based on incomplete or misunderstood clinical laboratory data.

Earlier diagnosis of Lyme disease typically has a strong prognosis for recovery with the recommended two week regiment of oral antibiotics [211]. However, if gone undetected, progression of Lyme disease can result in cellular damage and long term physical ailment [212]. This means the earlier and more accurately a *B. burgdorferi* infection can be detected and proper treatment initiated, the better the outcome and the less chance for unnecessary treatment due to either a false positive results using existing testing or more radical treatment based on incomplete diagnostic results. Current controversies surrounding diagnosis and treatment of Lyme disease highlights the importance for an improved more sensitive and more specific diagnostic [213]. iPCR was demonstrated to have superior sensitivity to two-tier testing with particular improvements for detection of early disease. The DOC IgG optimized iPCR also demonstrated increased specificity over two-tier testing resulting in fewer false positive results for the serum samples in the CDC Research Panel II. The results for iPCR are quantitative and unambiguous and eliminate the need for IgM analysis, which remains a controversial topic in Lyme disease diagnostic research. The potential to reduce analysis to a simple single tier will also reduce the cost and complexity of Lyme disease diagnosis, simplify test result analysis and provide a more timely analysis of samples reducing the time to treatment for individuals that test positive. Taken together, iPCR detection of Lyme disease has

demonstrated the capability to provide a more effective means of diagnosing Lyme disease.

Future Directions

Reagent Optimization

One of the strengths of our iPCR approach is the use of a liquid phase capture of host-generated antibodies using antigen coupled magnetic beads. To accomplish the conjugation, a single commercial kit chemistry was used based on an epoxy surface chemistry (Dynabeads Antibody Coupling Kit, Invitrogen, Carlsbad, CA). Additional commercial methods are available for linking ligands (antigens, antibodies, proteins, etc.) to solid supports such as magnetic beads using a number of different covalent linkages. The chemical reactions that facilitate ligand attachment are well characterized and proceed by attachment of biomolecules through common chemical groups.

The diagnostic performance of a coupled antigen can be affected by the type and number of linkages formed between the bead and the protein. For example, if the approach for linking the antigen to the bead adversely affects the structure of the antigen, it could limit its effectiveness as a capture molecule. This is particularly true for capture of antibodies generated against the secondary structure of the antigen. A loss of signal could also result if the coupled ligand leaches from the magnetic beads, which would also adversely affect the shelf life of prepared beads. It is important to consider whether conjugation chemistry introduces a charged group to the beads that could cause nonspecific binding of either proteins and/or the reporter

oligonucleotide to the beads. A final consideration is a linkage chemistry that could alter the structure of the beads such as promoting aggregation and/or adversely affecting their binding characteristics. These are all important considerations for design of antigen coupling to the magnetic beads that with testing of each functional group reactivity could provide for a more sensitive assay.

The types of functional groups found in antigenic proteins typically used for attachment to magnetic beads and available in a commercial format include primary amines, sulfhydryls and carboxylic acids [214]. The most common functional target for immobilizing protein molecules is the amine group (–NH2). This group exists at the N-terminus of each polypeptide chain and in the side chain of lysine residues. Due to its positive charge at physiological conditions (pH 7.0), primary amines are usually located on protein surfaces and allow for bead conjugation without denaturing the protein structure [215]. This is highly advantageous so as to minimize impacts on antigen secondary structure that could hinder antibody recognition. The limitation to the amine group coupling is that different antigens have different numbers of amine groups and hence it is difficult to maintain a uniform coupling efficiency across distinct antigens.

In addition to amines, the thiol group can be used for direct coupling reactions using sulfhydryl (–SH) groups which exist in the side chain of cysteine [215]. Cysteines are joined together between their side chains via disulfide bonds (–S–S–) and provide secondary and tertiary structure to proteins. As opposed to amine groups that can be directly coupled to beads,

sulfhydryl groups must be reduced to make them available for immobilization. Sulfhydryl groups typically are present in fewer numbers than primary amines providing a potential method of selective and directional immobilization. A repeated number of sulfhydryl groups (cysteine residues) could be added to the terminus of an antigen providing a conjugation that will likely orient every protein molecule in the same way on the beads. The only drawback to this type of coupling reaction is the need to reduce naturally occurring disulfide bonds that may be present in the primary sequence of the antigen to make sulfhydryl groups available for covalent attachment.

In addition to amines and sulfhydryl groups, proteins also have carboxyl groups (–COOH) that can also be utilized for conjugation to beads [215]. This is based on attachment at the C-terminus of each polypeptide chain and any amino acid residues of either aspartic acid or glutamic acid. Due to their charge and similarity to amine groups, carboxyl groups are usually found on the surface of proteins making them readily available for attachment. Carboxylic acids can be used to link antigens to commercially available beads through the use of an intermediary reaction. This method does typically require activation with a water-soluble cross-linker making this linkage a slightly more technically complex. The advantage to this method of antigen conjugation to beads is the resulting bead surface has a low non-specific binding of nucleic acids, which could minimize non-specific interactions with the iPCR reporter oligonucleotide. Overall, examination of different antigen conjugation methods has the potential to improve the existing iPCR protocol through reduction of the background signal with minimized

effects on the true signal thus providing overall increased sensitivity through reduction of non-specific interactions.

In addition to antigen coupling, the other aspect of iPCR reagent development that could be improved is assembly of the secondary antibody/oligonucleotide complex. The existing method utilizes a commercial process for conjugation of streptavidin to the secondary antibody using a proprietary one-step process that requires no downstream purification methodology (Lightning Link Streptavidin, Innova Biosciences, Cambridge, UK). The reporter oligonucleotide is synthesized with a terminal biotin group and a bridge between the two molecules is formed through the biotin-streptavidin interaction. Although successful, this method has the potential to introduce background signal based on unconjugated free oligonucleotide and/or reporter antibody lacking the oligonucleotide. A more efficient method would be to avoid the use of bridging molecules and directly conjugate the oligonucleotide and reporter antibody. This could be accomplished through a proprietary commercial synthesis (Chimera Biotec, Germany) or a commercially available kit based on incorporation of an amine group on the 5' or 3' end of the oligonucleotide followed by directed antibody conjugation (Innova biosciences, United Kingdom). Additional methods of oligonucleotide/antibody conjugation would require analysis to determine the effect on both the true signal and the background signal.

Decreasing Noise and Increasing Signal

Most diagnostic assays have an inherent signal to noise ratio. In immunological-based assays, the signal is generated from the binding of the reporter molecule to its specific target with the noise due mainly to the binding of the reporter to any non-specific targets such as the matrix or other proteins. The higher the signal to noise ratio, the more an assay can sensitively detect its target above the call threshold. All iPCR methods developed to date have noise due to inherent background amplification similar to the background observed with other immuno-specific methods (i.e., ELISA) [127]. Testing of our iPCR method (data not shown) was undertaken at the preliminary stages of assay development to both increase the signal and decrease the noise in our particular assay design (iPCR with magnetic beads, streptavidin/biotin bridged antibody/oligo). Different methods of bead blocking (Milk, blotto, BSA, commercial proprietary, etc.) were tested with no detectable change in background noise. Beads were also pre-incubated with naive serum from the same (mouse to mouse) as well as different species (rabbit to mouse) again with no detectable change in signal or background amplification. Additional experiments explored the potential for background contribution from unbound oligonucleotide and it was finally determined that, similar to other methods, the background signal in our iPCR assay predominated from non-specific binding of the secondary antibody to the magnetic beads.

One possible alternative approach for reducing the iPCR background signal that remains to be explored is to replace the oligonucleotide-labeled secondary antibody with a different type of reporter molecule. Protein A,

Protein G, Protein A/G hybrids and Protein L all bind antibodies with different affinity based on species and isotype. These proteins have been explored as antibody reporter conjugates for Lyme disease ELISA based detection in zoo animals, game animals and hunting dogs [216, 217] and have shown good specificity for other infectious diseases [218, 219]. With the current optimized DOC IgG iPCR protocol, the most likely candidate would be Protein A/G but all antibody binding protein family members could be tested for application to iPCR detection of host generated antibodies in human serum samples. In contrast to decreasing the background noise of the assay, increasing the positive signal has the potential to also provide a more sensitive test. This could be accomplished by exploring other more sensitive and more complex reporter systems such as gold nanoparticles [220], electroconduction [221] or surface plasmon resonance [222]. However, the most applicable to the existing protocol would be to expand on the number of *B. burgdorferi* peptides in the hybrid antigen. This would require mapping the immunodominant peptides on known *B. burgdorferi* antigens. Besides the VlsE (C6) and OspC (PEPC10) antigens, this has only been accomplished on limited *B. burgdorferi* antigens to date [223-226]. Future studies aimed at epitope mapping of the protein panel that performed well with human samples (Chapter 3 Table 4) has potential to provide additional peptides to enhance the existing DOC hybrid antigen. It is also possible to predict conserved peptide antigens based on *in-silico* alignment of gene sequences from multiple species/strains. For example, an alignment of DbpA sequences for a number of strains and species (Figure 18) permits the prediction of a potential conserved *B.*

burgdorferi peptide that could be seroreactive. Additional antigens known or suspected to be seroreactive in humans (Chapter 1) would also provide an additional source of potential targets.

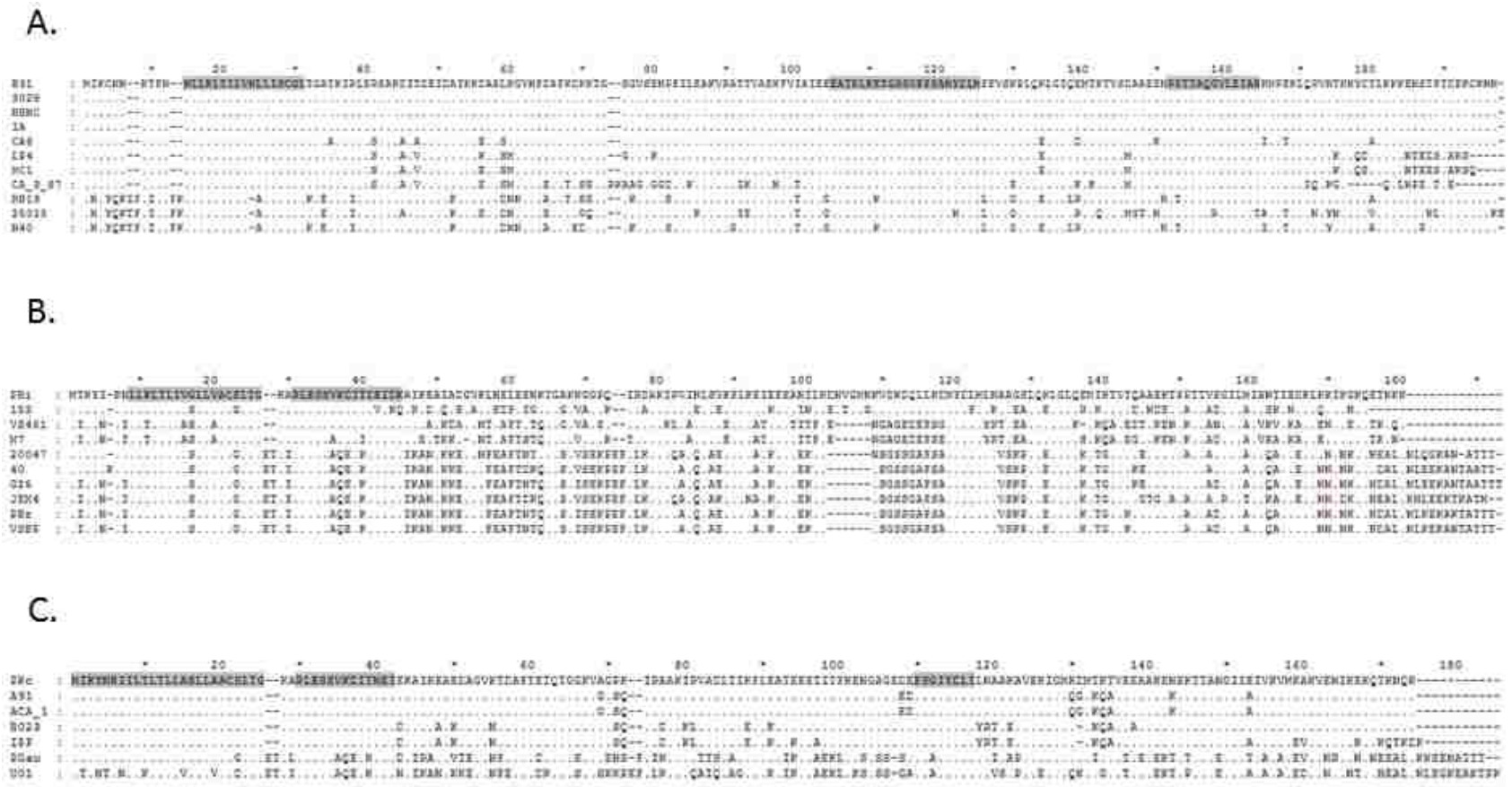


Figure 18. The DbpA antigen shows conservation at the *Borrelia* species level. An alignment of DbpA sequences from multiple strains for *B. burgdorferi* (top panel), *B. garinii* (middle panel) and *B. afzelii* (bottom panel) show regions of conservation for each species (gray highlight) that could function as peptide targets for further immuno-PCR development.

Additional Development and Testing

In addition to work aimed at improving the sensitivity and specificity of the iPCR assay, it is important to consider practical aspects related to the future clinical application of iPCR for routine diagnosis of Lyme disease. Additional development goals include: (1) increasing throughput, (2) more precisely defining measures of background, sensitivity and specificity, and (3) broadening the diagnostic capability of the assay.

The current protocol as published requires manual processing of individual samples. This not only provides for a more laborious and costly method but introduces the potential for human error. To increase throughput, a number of commercially available systems (plate washers, robotic liquid handlers, magnetic bead separators) have been developed for automated processing of magnetic bead based assays that can function using a 96-well plate format. A 96-well plate format would not only increase throughput but also provide more seamless compatibility with downstream qPCR analysis. Beyond commercially available units, there is the potential to automate the entire process in a microfluidic-type system that would combine both magnetic bead capture of host antibodies and PCR signal amplification in a single enclosed system. By increasing throughput, the efficiency of processing and more importantly repeatability would make transferring the protocol to a clinical setting more practical.

A higher throughput and more automated protocol will provide the opportunity to increase the numbers of samples to attain a statistically stronger measurement of the healthy human background to contribute to a

more accurate call threshold. It is also important to analyse an increased number of samples similar to the types already tested in the CDC research panels (Lyme early, Lyme late, look-alikes, healthy endemic and non-endemic) to provide a more robust estimate of the specificity and sensitivity of the iPCR assay. Increasing the number of Lyme patient and healthy samples tested will increase the overall confidence in assay performance and potentially move the assay closer towards clinical readiness.

Although it is important to continue to refine the iPCR approach for application to Lyme disease it is important to emphasize that the existing protocol is currently designed for testing of human samples suspected of infection with *B. burgdorferi*. Additional *Borrelia* species, *B. garinii* and *B. afzelii*, are known to cause Lyme disease in Europe and the amino acid sequences of the antigenic proteins from these species may be divergent from those of *B. burgdorferi* . Therefore, infections resulting from *B. garinii* and *B. afzelii* may not be detected with the existing DOC antigen IgG iPCR assay. Testing of the same antigens (DbpA, C6 and PEPC10) from *B. garinii* and *B. afzelii* could provide a useful diagnostic for European Lyme patients but would require similar validation testing to determine the sensitivity and specificity of the refined antigen targets. In addition to humans, domestic animals such as dogs and horses are also known to suffer from Lyme disease as a result of infection with *B. burgdorferi*. It would be important to determine whether the DOC IgG protocol developed for humans would be applicable to Lyme disease testing in animals. Using the existing protocol, this would simply require either replacing the secondary reporter antibody with a species

specific antibody or testing of the newly proposed protein A/G reporter system. With additional design and development studies, iPCR application for diagnosis of Lyme disease has broad potential as a diagnostic platform.

In total these future studies will provide insight into the applicability of iPCR for diagnosis of Lyme disease by optimizing the existing methods and expanding the protocol for more universal application with the goal of transferring the assay into a clinical setting for routine testing. This would help to improve diagnosis and ultimately treatment of a controversial and potentially debilitating infectious disease.

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